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ROLE OF T CELLS AND T CELL PRODUCTS IN PLACENTAL PROLIFERATION AND FUNCTION DURING MURINE PREGNANCY

BY

© IRENE ATHANASSAKIS - VASSILIADIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (IMMUNOLOGY)

EDMONTON, ALBERTA
SPRING, 1988

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ABSTRACT

The placental immunotrophism hypothesis, which postulates that maternal T cells are stimulated by fetal alloantigens in the placenta to secrete their products to improve placental growth and fetal viability was evaluated by the present work, which was based on three predictions from this hypothesis: (a) T cell products stimulate trophoblast growth; (b) maternal T cell depletion *in vivo* decreases placental growth; and (c) relevant lymphokine production can be found in the decidua. The results showed that the T cell-derived lymphokines IL-3 and GM-CSF, as well as the cytokine CSF-1, stimulate fetal-adherent phagocytic, non-specific esterase-positive placental cells to proliferate *in vitro*. These cells were identified as trophoblasts, by the criteria of being cytokeratin-positive and vimentin-negative. They are also macrophages, by the criteria of F4/80 and Mac-1 positivity, and we therefore call them "trophoblast-macrophages". The second prediction from the immunotrophism hypothesis was evaluated by *in vivo* depletion of maternal T cells using specific monoclonal antibodies (anti-CD4 and anti-CD8). Mid-gestational injection of anti-CD8 antibody into allogeneically or syngeneically pregnant females decreased placental proliferation and phagocytosis in both cases. Treatment with the anti-CD4 monoclonal antibody showed the same effect on placental proliferation, but did not affect

placental phagocytosis. Interestingly, simultaneous injection of anti-CD4 and anti-CD8 antibodies lowers phagocytosis in the allogeneic but not the syngeneic situation. Finally it was shown that growth factors capable of stimulating placental cell proliferation were present in decidual cell supernatants. These supernatants were able to induce proliferation of the DA-1 cell line, which is known to respond to IL-3 and GM-CSF. Allogeneic decidual supernatants were more stimulatory of DA-1 cells than syngeneic supernatants and the appearance of stimulatory ability paralleled the influx of lymphocytes into the decidua. Growth factor production by decidual cells depended on T cells, since maternal T cell depletion abolished the stimulatory activity of decidual supernatants, when tested on DA-1 cells. The active lymphokine in the decidual preparations was shown to be GM-CSF, since anti-GM-CSF antibody almost completely blocked its activity. Thus, the results presented in this thesis provide evidence in support of the placental immunotrophism hypothesis.

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GLOSSARY

blastocyst: the modified cavitated stage of mammalian embryos, consisting of the inner cell mass, and a thin outer trophoblast layer, the trophectoderm (day 3.5 of gestation).

chorion: membrane enclosing the entire conceptus, derived from fetal cells (somatopleur or ectoderm plus mesoderm).

cytokeratins: proteins which are members of a multigene family present only on epithelial cells.

cytokines: glycoprotein mediators secreted by any type of cell.

decidua: the area of endometrium between the implanted chorionic vesicle and the myometrium, which becomes the maternal part of the placenta.

endometrium: the mucosal membrane lining the uterus.

ectoplacental cone: the actively growing part of the trophoblast involved in the formation of the placenta in rodents (day 7.5 of gestation).

lymphokines: biologically active, soluble mediators produced by T cells upon stimulation.

morula: the mass of blastomeres resulting from the early cleavage divisions of the zygote which ends at cavitation or blastocyst stage.

ovum: ovulated mature female gamete.

placenta: the organ of communication between the mother and the fetus. *hemochorial placenta*: a type of placenta as in man and rodents, in which three layers of cells (villous epithelium, connective tissue and endothelium of the fetal blood capillaries) are interspersed between maternal and fetal blood streams.

trophoblast: the ectodermal cell layer covering the blastocyst which in some species erodes the uterine mucosa and through which the embryo receives nourishment from the mother. Trophoblast cells are restricted to the extra-embryonic tissues and contribute to the formation of the placenta.

trophectoderm: the outermost layer of cells in the mammalian blastocyst.

vimentin: the main subunit protein of intermediate filament in endothelial cells.

ABBREVIATIONS

ADCC: antibody dependent cell mediated cytotoxicity

AFP: alpha-fetoprotein

ATS: anti-thymocyte serum

BCDF: B cell differentiation factor

BCGF: B cell growth factor

ConA: concanavalin A

CSF-1: colony stimulating factor 1

CTL: cytotoxic T lymphocytes

EPC: ectoplacental cone

FAPP: fetal adherent phagocytic placental cells

GM-CSF: granulocyte-macrophage colony stimulating factor

GPI: glucose phosphate isomerase

hCG: human chorionic gonadotropin

^{3}HTdR: ^{3}H-thymidine

ICM: inner cell mass

γ-IFN: γ-interferon

IL-2: interleukin 2

IL-3: interleukin 3

MHC: major histocompatibilty complex

MLR: mixed lymphocyte reaction

OD: optical density

PHA: phytohemagglutinin

PMA: phorbol myristate acetate

CHAPTER I

INTRODUCTION

The implantation of the early embryo in the endometrium and the development and survival of the feto-placental unit during gestation reflects a highly successful process of allotransplantation. The absence of vascular continuity between mother and fetus and the efficiency of the placenta in restricting, if not entirely preventing, the transfer of immunocompetent cells to the fetal circulation, places the fetus in a very different category than any surgically transplanted tissue or organ. The genetically disparate components of the conceptus that are exposed to the maternal host are restricted to the trophoblast and the extra-embryonic membranes. Therefore, beginning with a description of the functional anatomy of the placenta, this section will concentrate on the antigenicity of the feto-placental unit, and on how this influences the maternal immune system.

I) ANATOMY OF THE PLACENTA

After fertilization, the ovum undergoes holoblastic cleavage, becoming a solid sphere of cells called the morula, which then gives rise to the blastocyst.The outer layer of blastocyst, the trophectoderm, gives rise to membranes that surround the embryo, while the embryo proper forms from the inner part of the blastocyst, the so-called inner cell mass (Gardner et al. 1973). At the blastocyst stage the embryo implants itself into the endometrium on the wall of the uterus, where it remains and is nourished until birth.

In the murine blastocyst, the trophectoderm in the abembryonic and lateral areas gives rise to primary trophoblastic giant cells. Their function is not fully established but probably includes an anchoring mechanism during the early stages of implantation. The trophectoderm overlying the inner cell mass at the embryonic pole of the blastocyst undergoes rapid proliferation to form the so-called ectoplacental cone, comprised of highly invasive trophoblast cells. From its superficial regions arise the secondary trophoblastic giant cells that migrate outwards, to lie at the margins of the developing placenta, and downwards to surround the early embryo. These cells become highly polyploid by a process of endomitosis (Chapman et al. 1972) and their function is obscure. The proliferating centre of the ectoplacental cone produces tissue that differentiates to form the major fetal components of the placenta, the labyrinthine trophoblast and the spongiotrophoblast (Fig. 1). The giant cells and the spongiotrophoblast abut uterine decidual tissues with close cellular contact. In the labyrinthine trophoblast (Fig. 2) the tips of the villi fuse, the fetal vessels anastomose and the outer layer is bathed by maternal blood in the placental sinuses.

After implantation the human blastocyst gives rise to two forms of trophoblast, the inner cellular cytotrophoblast and the outer syncytiotrophoblast (Fig. 3). The syncytial form is derived by differentiation accompanied by cell fusion and increases throughout pregnancy. At an earlier stage, columns of cytotrophoblast push through the syncytium, forming the villous primordia and spread out at the ends to form a shell around the embryo. The characteristic mature placental villi develop ultimately with an outer covering of syncytium and an underlying layer of cytotrophoblast which, in the later stages of pregnancy, becomes discontinuous. It is the villous syncytium that is bathed in the maternal blood, and it is the trophoblast of the basal plate that is in

FIGURE 1

Diagrammatic representation of a saggital section of the definitive mouse placenta. UW=uterine wall, DC=decidual cap, SP=spongiotrophoblast, LAB=labyrinthine trophoblast, AM=amnion, EM=embryo.

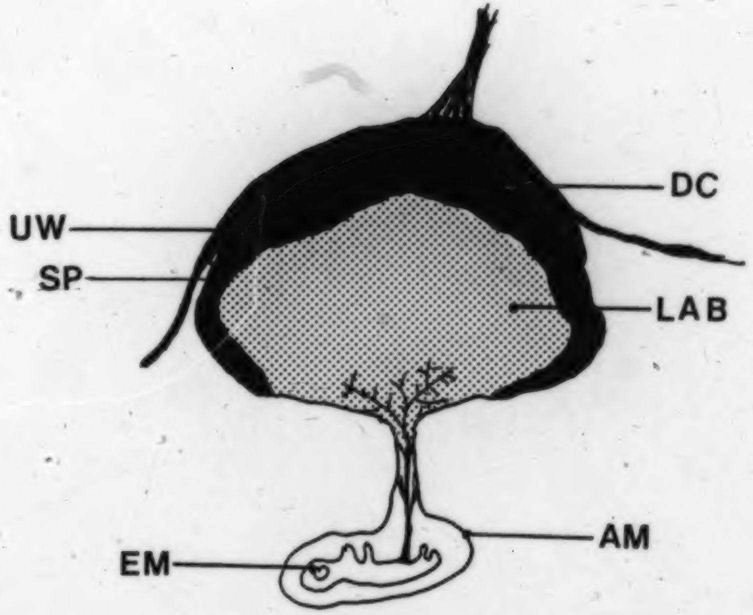

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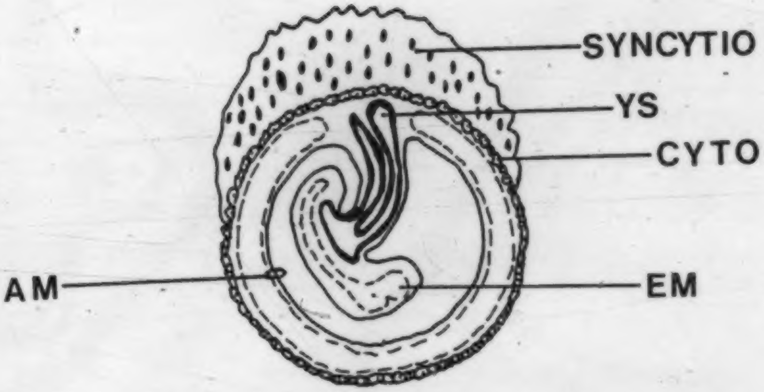
FIGURE 2

Placental labyrinthine trophoblast on day 12 of gestation in the mouse (C3H/HeJ female mated to BALB/c male). MC=maternal blood cells, FC=fetal blood cells. Arrows show some trophoblasts. Magnification x40x3.3.

FIGURE 3

Cellular and syncytial forms of trophoblast in the 8 week old human embryo. SYNCYTIO= syncytiotrophoblast, CYTO=cytotrophoblast, YS=yolk sac, AM=amnion, EM=embryo.


SYNCYTIO
YS
CYTO
AM
EM

close contact with the uterine decidual tissue in the organized placenta. The chorionic membrane, composed mainly of a layer of cytotrophoblast, surrounds the remainder of the conceptus after the establishment of the main placental region.

The hemochorial placenta is a complex organ containing different types of trophoblast and a variety of other fetal tissues such as endothelium and fetal blood elements. In addition, there are elements provided by the maternal tissues including decidua, connective tissue, and blood cells. Immunological studies have often neglected the presence of these maternal cell populations and questionable conclusions have been drawn as to the properties of trophoblasts. The isolation of pure trophoblast has been the subject of intense investigation. Rossant and Croy (1985), using embryo transfer and reconstitutive blastocyst techniques, combined with isozymal and *in situ* genetic markers, have established that approximately 70% of the 13-15 day murine placenta is trophectoderm derived, 30% is maternal in origin and 4% develops from the inner cell mass. Recently Zuckermann and Head (1986a), using step gradient separations of murine placental cells, isolated a cell population with trophoblastic characteristics. These include cytokeratin-positive and vimentin-negative expression, growth as a monolayer of polygonal cells, and the ability to give rise to mono- and multi-nucleated giant cells able to secrete progesterone. Some of these cells express alkaline phosphatase and are phagocytic. The majority is non-specific esterase negative. These results show that trophoblasts consist of heterogeneous cell populations whose identity is not yet clear. At the later stages of pregnancy the direct contact of the murine spongiotrophoblast (and human cytotrophoblast) with maternal tissues indicates these to be the most likely sites of maternal immune recognition of fetal alloantigens. The nature of the fetal alloantigens as well as their localization in the intact placenta will be discussed next.

II) ANTIGENIC EXPRESSION OF THE PLACENTA

Transplantation antigens encoded by genes of the major histocompatibity complex (H-2 in mice and HLA in humans) trigger graft rejection. Pregnancy is the only physiological situation known where the allograft survives despite the presence of class I antigens in direct contact with allogeneic immunocompetent cells. This section is focussed on the expression, localization and nature of transplantation antigens on the feto-placental unit.

A) CLASS I ANTIGEN EXPRESSION ON THE PLACENTA

The development of sensitive immunoassays has resolved many controversies on the distribution of class I MHC antigens in the feto-placental unit. There is now a general concensus that class I antigens are synthesized and expressed only at low levels on the trophectoderm (Webb et al. 1977, Goldbard et al. 1984). These antigens disappear after implantation, (Searle et al. 1976, Billington et al. 1977) and are detected on or after day 6 of pregnancy on parietal endoderm and Reichert's membrane, but not on the tissues of the embryo itself (Heyner 1973). Class I antigens are first expressed on the nine to ten day embryo, but this expression varies in the different parts of the body and is strain-dependent (Kirkwood and Billington 1981, Bell and Billington 1983). Placental cells from day ten to seventeen of gestation show increased expression of class I antigens, since the tissue is able to specifically absorb paternal anti-class I antibodies from the circulation of pregnant mice (Wegmann et al. 1979a, 1979b, Singh et al. 1983).

Class I antigen expression is restricted to certain trophoblast populations. The absence of detectable class I antigens on the human chorionic villi (Faulk and Temple 1976) was extrapolated to all trophoblast populations. However, it is now clear that HLA class I antigens are expressed on those trophoblast cells in direct contact with maternal tissues (blood and decidual cap) (Sunderland et al. 1981, Montgomery and Lala 1983, Redman et al. 1984, Butterworth et al. 1985). In the mouse and rat, placental class I positive cells are localized in the spongiotrophoblast (Jenkinson and Owen 1980, Colavincenzo and Lala 1985, Singh et al. 1983, Billington and Burrows 1986a, Ho et al. 1987). Although some investigators reported that labyrinthine trophoblast is class I positive (Chattergee-Hasrouni and Lala 1982), their results could not be reproduced (Singh et al. 1983). Another unusual observation was made in baboon placentae, where, using monoclonal antibodies to human MHC determinants, Stern et al. (1987) detected class I antigens on the villous syncytiotrophoblast. However, in these experiments the possibility of cross-reactivity with non-MHC baboon antigens has not been ruled out. In fact, the labyrinthine trophoblast in the mouse placenta, or the syncytiotrophoblast in the human placenta, may express other unusual class I MHC antigens that the reagents and techniques used in prior studies could not detect (Hunziker 1987).

In at least some species, the class I antigens expressed on trophoblast subpopulations do not display classical MHC structures. Immunoprecipitation experiments showed in both humans and baboons that the heavy chain of class I antigens on the placenta has a lower molecular weight when compared to the classical MHC molecules, but associates normally with beta$_2$-microglobulin (Stern et al. 1987, Ellis et al. 1986). The rat and mouse placentae appear to bear both classical and unusual class I antigens (Billington and Burrows 1986a, Ho et al. 1987, Hunziker 1987). The spongiotrophoblast of rat placenta expresses an apparently unusual class I antigen, the *Pa* antigen which, however, has the classical molecular weight (Ho et al. 1987, MacPherson et al. 1986, Ghani et al. 1984). Murine trophoblast cells can specifically compete with conventional tumor target cells in an alloreactive T-cell-mediated cytotoxicity assay (Zuckermann and Head 1987a), which provides evidence for the expression of classical MHC antigens on the surface of these cells. Dot blot hybridization techniques of placental extracts showed that mRNAs encoding both classical and non-classical class I antigens from the K, D, L and TL regions are expressed during mid and late gestation in the mouse placenta (Hunziker 1987). The expression of fetal class I antigens in the placenta should make this tissue antigenic. Indeed when placental or fetal cells are injected into virgin females they can induce alloantibody production (Billington 1986b). The placental cells, but not fetal cells, stimulate an antibody isotype distribution akin to that seen in allogeneic pregnancy, suggesting that maternal immune cell recognition of allogeneic placental rather than fetal cells is the basis of maternal immmunity during pregnancy.

B) CLASS II ANTIGEN EXPRESSION ON THE PLACENTA

Preimplantation blastocysts and post-implantation embryos (day 7.5) are uniformly negative when tested with anti-Ia antiserum. Trophoblasts are still class II negative even after IFN-treatment (Zuckermann and Head 1986b). Immunoprecipitation methods revealed that Ia antigens first appear on the fetus on day eleven of gestation and are restricted to the liver until day sixteen (Delovitch et al. 1978). Immunohistological studies suggest that no human trophoblast population normally expresses class II antigens such as HLA-DR or DQ (Bulmer and Johnson 1985).

However, in late-stage placentae, HLA-DR positive cells have been identified in the subperipheral layer of the amnion, and, sparsely, within the chorionic villous stroma (Sutton et al. 1983). These cells are probably fetal dendritic cells or macrophages and are not exposed to the maternal circulation (Sutton et al. 1983, Bulmer and Sunderland 1984a, Bulmer and Johnson 1984b). Macrophages have also been identified in late-stage murine placenta (day 14 to 18 of gestation), in yolk sac membranes and in pregnant uteri (Wood 1980; Hunt et al. 1985). Although the origin of these macrophages is not clear, it has been postulated that their role is to filter out potentially harmful anti-fetal antibodies during passage of immunoglobulin from the mother to the fetus (Wood et al. 1978). The distribution of Ia-positive cells may be important in the generation of an immune response towards the fetus since an antigen must be recognized by immunocompetent cells in association with an Ia molecule in order to generate a response.

III) REGULATION OF MATERNAL IMMUNITY DURING PREGNANCY

During pregnancy, many immunological changes take place in the maternal system. Specific populations of immune cells increase in number both systemically and locally. At the level of the fetal-placental environment, either stimulatory and/or suppressor immune mechanisms or the mechanical barrier of the placenta are critical in protecting the fetus.

A) CHANGES IN MATERNAL LYMPHOCYTE SUBSETS

During pregnancy, there is an increase of certain hormones, notably adrenal corticosteroids, and an involution of lymphoid tissues, mainly the thymus, which in the mouse may loose 70% of its initial weight (Beer and Billingham 1976, Maroni and DeSousa 1973, Nelson and Hall 1964). The thymus is a steroid-responsive tissue and the changes in steroid blood levels during pregnancy can alter the release of thymic humoral factors and the differentiation of precursor T cells in the maternal and fetal thymus (Stimson 1983). Indeed, thymic Ly1+,2-bearing populations drop markedly during gestation. This could be explained by the thymic involution resulting primarily from the loss of immature cortisone-sensitive lymphocytes (Mathieson et al. 1979). The tissue distribution of maternal lymphocytes changes at different times during pregnancy (Chatterjee-Hasrouni et al. 1980).

The temporal patterns of change in the absolute number of lymphocytes in various lymphoid organs are qualitatively similar in both syngeneic and allogeneic pregnancy but the changes are more prominent in the allogeneic situation. In pregnant females, Ly2-positive and I-J bearing lymphocytes, which mark the appearance of suppressor T cells, increase during pregnancy as compared to virgin females (Taniguchi et al. 1980; Cantor and Boyse 1975; Chaouat and Voisin 1981; Lala et al. 1983). Successive waves of null cells increase in the bone marrow (day 6 to 8), blood (day 8 to 9), spleen and para-aortic lymph nodes (day 12 to 14). In contrast, B cell numbers remain essentially unchanged during pregnancy (Lala et al. 1983). These changes may reflect the recognition and response to an embryonic antigen. Alternatively, the more dramatic increase of these cell subsets seen during allogeneic pregnancy may represent a response to the paternal alloantigens. Similar responses have been seen in tumor transplanted mice (Lala and Kaizer 1977, Lala and McKenzie 1982). A small increase in splenic NK activity (Lala et al. 1983) may be explained by the rise in interferon levels during gestation (Fowler et al. 1980). This may represent an adaptive cell-mediated response to the oncofetal-type antigens on the placenta, without any harmful effects on the conceptus.

Changes in the immune cell populations also occur locally in the decidua, which is the maternal tissue that is in closest contact with the fetal placenta. The total cellularity of the decidua, including lymphocytes, peaks on days 11 and 15-16 of pregnancy and the cell accumulation is higher in allogeneic versus syngeneic pregnancy (Lala et al. 1986). Null cells account for the majority of lymphocytes in this tissue. Although the frequency of B cells is low, Thy-1+ cells and especially the Lyt1+2- phenotype have the same frequency as that in the blood. Late allogeneic decidua show an increase of Ly2+1- cells. In addition Mac-1 positive cells reach maximal numbers by day 12 of pregnancy (Lala et al. 1986). Since T cells produce many factors able to stimulate cell proliferation, the next section will briefly describe the major lymphokines and the cytokine CSF-1, as possible candidate molecules involved in the maternal-fetal interaction in the mouse.

B) LYMPHOKINES AND CYTOKINES

Lymphokines and cytokines are specific regulatory glycoproteins the first secreted exclusively by immune cells, whereas the latter can be produced by any other cell type. Some lymphokines, such as GM-CSF and IL-3, are identified because of their ability to stimulate precursor cells to form colonies of progeny cells in semi-solid medium and are referred to as colony-stimulating factors (CSFs). The best characterized lymphokines are the CSFs controlling granulocyte-macrophage populations and the T cell regulators, Interleukin 2 (IL-2) and Interleukin 1 (IL-1), whereas the activators of the B cell lineage (eg, B cell growth factor) and the eosinophil series (eosinophil differentiation factor) are less well characterized (Metcalf 1986). In this section I will concentrate on the description of the murine lymphokines and cytokines used in the present study.

INTERLEUKIN 3: The only normal cell type so far documented as being able to synthesize interleukin 3 (IL-3) or multi-CSF is antigen- or mitogen-primed T cells (Schrader et al. 1981, Schrader and Iscove 1980). However, IL-3 is also produced constitutively by the myelomonocytic leukemic cell line WEHI-3B and by a T cell hybridoma (Fung et al. 1984, Yokota et al. 1984). The mature molecule of IL-3 contains 140 amino acids and has an approximate molecular weight of 15,000 daltons. The IL-3 gene exists as a single copy, has five exons and maps on chromosome 11 (Campbell et al. 1985, Miyatake et al. 1985). In mouse IL-3 is known to stimulate granulocyte and macrophage colony formation (CFU), colony formation by multipotential cells, BFU-E, CFU-E, eosinophil and megacaryocyte progenitors and the proliferation of mast cell lines and

IL-3 dependent hematopoietic cell lines (Metcalf 1986).

GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR: Mouse granulocyte-macrophage colony stimulating-factor (GM-CSF), which stimulates granulocyte and/or macrophage colony formation, was initially purified from mouse lung conditioned medium (Burgess et al. 1977), and later was found to be synthesized primarily by T cells (Kelso and Metcalf 1985). It is a glycoprotein with a molecular weight of 15,000 daltons and contains 124 amino acids. It exists in a single-copy genomic form and maps to chromosome 11 (Gough et al. 1984). GM-CSF is known to stimulate granulocyte and macrophage colony formation and at high concentrations, eosinophil, megacaryocyte, and erythroid colony formation (Metcalf 1986).

INTERLEUKIN 2: This is a glycoprotein with a molecular weight of 23,000 daltons. The IL-2 gene maps to chromosome 12, exists as a single-copy, and has four exons (Fuse 1984). It is produced by T lymphocytes (Smith 1984, Paetkau et al. 1985), and is characterized by its ability to stimulate the proliferation of activated T lymphocytes. It regulates cellular immune responses and may replace helper T cells in the generation of cytotoxic T lymphocytes, augment natural killer cell activity (Henney et al. 1981), generate lymphokine-activated killer cells (Grimm et al. 1982) and B cell growth and differentiation

(Kishi et al. 1985, Mond et al. 1985). This is the best characterized lymphokine, not only with respect to its molecular biology but to that of its cellular receptor as well. The IL-2 receptor has a molecular weight of 55,000 daltons and forms heterodimers which might be necessary for the formation of high affinity receptors (Robb et al. 1987, Neeper et al. 1987).

COLONY STIMULATING FACTOR 1: This glycoprotein is a product of fibroblasts, embryonic yolk sac, whole embryo, and pregnant mouse uterus. Colony stimulating factor 1 (CSF-1) is a dimer of two identical subunits each having a molecular weight of 14,000 daltons. It exists in a single-copy genomic form but in several messanger RNA species (1 to 4 kb, Wong et al. 1987). This cytokine specifically stimulates the formation of macrophage colonies (Guilbert 1985).

The changes in the various maternal-lymphocyte subsets during both syngeneic and allogeneic pregnancy suggest that the mother becomes fully cognizant of the conceptus in her reproductive tract, and responds to embryonic antigens as well as to paternally-derived alloantigens expressed at the maternal-fetal interface. The questions that naturally arise are: how do these lymphocyte subset changes reflect the functional aspects of the maternal immune response against the fetus and what role might these changes have in fetal survival? The mechanisms provided by both maternal and fetal systems to ensure fetal survival will be discussed in the following section.

IV) MECHANISMS CONCERNING THE SURVIVAL OF THE FETAL ALLOGRAFT

In the literature of reproductive immunology, there are two major aspects of the survival of the mammalian fetal allograft. The first deals with the mechanisms that the fetus provides for its own protection which are concentrated on the role of placental barrier. The second aspect deals with the maternal immune mechanisms elaborated to protect not only fetal survival but the maternal system as well. I will now examine each in turn.

A) THE PLACENTA AS AN IMMUNOLOGICAL BARRIER BETWEEN THE MOTHER AND THE FETUS

The first clear experimental indication that the trophoblast plays a crucial protective role in the survival of the fetal allograft came from the early experiments of Simmons and Russell (1962). They found that, although the embryonic component of the fetoplacental unit is rejected as an allogeneic graft, the trophoblastic component is completely untouched by allogeneic rejection mechanisms. Maternal humoral immune responses directed against the paternally inherited antigens of the genetically alien embryo have been detected in several species including man, horse, cow, rat, mouse and salamander (Billington et al. 1983), either as free alloantibodies or as soluble immune complexes.

Early experiments by Swinburne (1970) indicated that the placenta could serve as an immunoabsorbent barrier for maternal anti - paternal MHC antibodies made during allopregnancy. More recently, experiments using radiolabelled monoclonal anti-fetal antibodies against class I, showed that these antibodies will bind specifically (Wegmann et al. 1979a,b, 1980; Chaouat et al. 1983a) and not to Fc receptors present on cells in the placenta (Tyan 1975), in two major sites: one in the lateral aspect of the placenta where the yolk sac joins the fetus and a second site in the region of spongiotrophoblast (Singh et al. 1983). These antibodies, which show a rapid turnover in the placenta, are digested intracellularly and then released as fragments back into the circulation of the pregnant female (Raghupathy et al. 1981, Raghupathy 1982). These studies suggest that the placenta can serve as an immunoabsorbent barrier to antibodies directed against the paternally derived class I MHC antigens which are in direct contact with the maternal circulation. Although some trophoblast populations express class I MHC antigens, it is generally accepted that trophoblasts are not susceptible to lysis by allospecific CTL, activated natural killers, natural cytotoxic cells, ADCC or alloantibody and complement treatment. Early studies showed that pre- and post-implantation embryonic and extraembryonic tissue can survive when transplanted into ectopic sites in allogeneic recipients (Simmons and Russel 1962, Kirby et al. 1966). However, when the recipients were preimmunized, only the ectopically transplanted ectoplacental cone (EPC) survived, whereas blastocysts failed to grow. This has been subsequently verified by *in vitro* experiments in which allogeneic CTL or alloantibody and complement could not kill EPC-derived trophoblast cells (Vandeputte and Sobis 1972, Jenkinson and Billington 1974). Other studies have shown that when trophoblasts from the mature murine placenta have been stimulated with γ-IFN to increase class I expression, they become susceptible to lysis by anti-paternal or heterologous antisera and complement, whereas they are not affected by class I specific CTL (Zuckermann and Head 1987a, Zuckermann and Head 1987b). In addition, trophoblasts can specifically compete with tumor cell targets which are otherwise susceptible to lysis by cytotoxic lymphocytes, i.e., they can serve as cold target inhibitors (Head et al. 1987). The relative resistance of trophoblast cells to lysis by antibody conforms to the expectation posed by the barrier hypothesis, namely that no harmful anti-paternal antibodies can cross the placenta and reach the semi-allogeneic fetus. A question then arises whether the placenta is also a barrier to maternal cells. The passage of sensitized cells from the mother to the fetus through the placenta is controversial and is usually associated with fetal abnormalities. Collins et al.(1981), using MHC antigens as markers, reported that 30% of the dividing cells in the liver of the newborn are of maternal origin. However, other investigators, using the glucose phosphate isomerase (GPI) isozyme marker, which can distinguish as little as 1% maternal cells mixed with 99% fetal cells, failed to confirm these reports (Hunziker et al. 1984). In these studies Hunziker at al. (1984) have shown that significant numbers of maternal cells were detected only in 2 of the 172 offspring tested, and that although some maternal red cells cross the placenta, maternal white cells are rarely found in the fetus.

B) THE ROLE OF MATERNAL IMMUNE MECHANISMS IN FETAL SURVIVAL

Different types of immune-suppressive activity have been studied in pregnant females. Pregnancy-associated substances suppress alloreactivity in mixed lymphocyte culture (MLC) *in vitro* (pregnany-associated alpha-2 glycoprotein Stimson 1972, alpha-feto protein Murgita and Tomasi 1975, human chorionic gonadotropin Stimson 1983), prolong allograft survival (progesterone Munroe 1971) and depress lymphocyte proliferation induced by mitogens (pregnancy-associated alpha-2 glycoprotein Stimson 1976a, alpha-feto protein Gupta and Good 1977, pregnancy-specific beta-1 glycoprotein Honre et al. 1976, human placental lactogen Cerni et al. 1977, human chorionic gonadotropin Stimson 1983). Placental cell products may also exert a direct inhibitory effect on lymphomyeloid cells and may directly block the interaction between effectors and their targets in the CTL response, natural killer cell assays, and antibody-dependent cell mediated cytotoxicity (ADCC) (Chaouat et al. 1979, Fauve et al. 1984).

Maternal anti-paternal antibody may also exert an immunosuppressive effect. Antibodies eluted from murine placentae and administered to naive recipients allow the growth of paternal strain tumors (Voisin and Chaouat 1971). The effect has been ascribed to the masking of antigenic determinants relevant to the stimulation of host immunity (Chaouat et al. 1979). Immune complexes are known to induce a suppressive state which may act on different levels of the immune response: e.g. antigen masking, through interaction with both antigen and Fc receptors on B cells, through interaction between Fc receptors on B cells (antigen non - specific), effector cell blockade, activation of suppressor T cells, blockage of antigen receptor on T cells, blockage of T and B cell interaction, inhibition of ADCC, blockage of cell-mediated lymphocytic reactions or blockage of delayed hypersensitivity (Theofilopoulos et al. 1981). During pregnancy, anti-paternal antibodies are found to specifically inhibit

MLR against paternal lymphocytes, lymphokine release and generation of CTL *in vitro* (Chaouat et al. 1979, Robert et al. 1983, Bonneau et al. 1973). These antibodies are primarily of the non-complement fixing IgG1 isotype (Bell and Billington 1980, Stewart et al. 1984).

Lymphocytes bearing markers specific for suppressor cells have been found systemically in the gestating female (Tanigushi et al. 1980, Lala et al. 1983, Cantor and Boyse 1975, Chaouat and Voisin 1981, Sano et al. 1984). These suppressor cells are paternal MHC-restricted and Lyt 2 positive. They are found in the spleen, peripheral lymph nodes and blood, and their appearance is usually delayed until after the first allopregnancy (Chaouat and Voisin 1984, Nagarkatti and Clark 1983). These suppressor cells elaborate a soluble antigen-specific suppressor factor *in vitro* which inhibits the generation of CTLs (Nagarkatti and Clark 1983) and the MLC reaction (Engelman et al. 1978). However, this suppression is not strong enough to prevent rejection of grafts of MHC incompatible paternal tissue in a primary or secondary response (Chaouat and Voisin 1981, Nagarkatti and Clark 1983) and cannot prevent rejection of paternal tumor grafts placed within the uterus of alloimmunized mice (Nagarkatti and Clark 1983).

Another type of non-T suppressor cell can be identified during pregnancy which is non-MHC restricted and is concentrated in the decidua at implantation sites, and in draining lymph nodes of the uterus (Clark et al. 1983). This suppressive activity is associated with cells that have cytoplasmic granules, but are not NK cells, and is mediated by a soluble factor which inhibits the generation of CTL (Clark et al. 1980). This local immunosuppression suggests a role for the decidua in prolonging fetal allograft survival. In addition it has been demonstrated that the absence of these cells correlates with abortion (Clark et al. 1983, 1984). These investigators observed that CBA females pregnant by DBA/2 males show an increased spontaneous abortion rate, which correlates with the absence of suppressor activity in the decidua. Furthermore, immunization of the CBA females with BALB/c spleen cells prior to mating by DBA/2 males lowered the resorption rate and increased decidual active suppression (Chaouat et al. 1983). These observations suggest that suppressor T cells should have an important role in preventing the development of harmful types of anti-fetal immune responses as a consequence of successive matings.

Another type of experimental evidence correlates the generation of maternal immune response with the increase of placental size and fetal viability. Early observations indicate that F1 hybrid murine placentae are larger than those of either inbred parental strains (Billington 1964). Further there have been reports that females immune to paternal antigens produce larger placentae than tolerant females (James 1965,1967). Therefore Clarke and Kirby (1966, 1967) postulated that maternal immune recognition leads to improved fetal viability and therefore provides a mechanism that could explain at least some of the extensive polymorphism that exists in the MHC locus. These observations are directly analogous to experimental situations where tumor development is stimulated rather than inhibited by an immune response (Fenderson et al. 1983; Prehn and Lappe 1971,Prehn 1983). Experimental situations focusing on the nature of the uterus as an immunologically unique site of implantation have shown that more embryos develop in a uterine horn that has been sensitized against paternal antigens with local skin grafts and cell challenges than in the contralateral unsensitized horn (Billingham 1971, Beer and Billingham 1974). However, there is an opposite school of thought which states that all of the advantages of the hybrid fetus are due to heterosis and not to activation of the immune system (McLaren 1975, Finkel and Lilly 1971, Hetherington et al. 1976, Hetherington and Humber 1977). Although we lack controlled experimentation, in man there is evidence that immunologic factors with regard to maternal-fetal incompatibility, influence placental growth. Warburton and Naylor (1971) have shown that a large increment in placental weight in normal pregnancies occurred between the first and the second birth. However their "mate-change" group failed to show this increment. This suggests that the smaller placental weights in a second pregnancy with dissimilar mates can be attributed to the absence of prior sensitization by paternal antigens. Blood groups may also make a difference in human pregnancy. Group O mothers have generally the smallest placentae whereas AB mothers have the largest (Jones 1968).

Recent observations on spontaneous abortions in humans have renewed the idea that maternal immunization with paternal alloantigens leads to improved fetal viability. Women undergoing multiple spontaneous abortions with no obvious chromosomal, endocrinological or gynecological abnormalities carry pregnancy to term if first immunized with paternal or third-party leucocytes (Beer et al. 1981, Taylor and Faulk 1981, Johnson et al. 1984, Mowbray et al. 1985a). Unfortunately, these trials, with the possible exception of the trial reported by Mowbray et al. (1985a), lack the proper double-blind clinical controls so that placebo effects cannot be ruled out. In some of these studies, such couples showed significantly more sharing of HLA specificities than do control groups (Kolmos et al. 1977, Beer et al. 1981, Gerencer et al. 1978, 1979, Gill 1983, Taylor and Faulk 1981). The HLA sharing in couples experiencing spontaneous abortion of unknown etiology correlates with a marked alteration in the mixed lymphocyte reaction between partners when compared with MHC typed third-party stimulators and responders (Beer et al. 1981), as well as with the absence of IgG blocking antibody (Takeushi 1980). Immunization of chronic aborters with paternal or third-party lymphocytes enabled the completion of gestation and correlated with the generation of anti-paternal antibody. However, some of the offspring of the chronic aborters who conceived under immunotherapy developed intrauterine growth retardation, SCID or chromosomal abnormalities (Beer et al. 1986). Gill has suggested that the failure of these conceptuses is due to the action of homozygous recessive lethal genes which escape elimination under the protection of blocking antibodies produced after treatment (Gill 1983). In contrast, other investigators have not found increased sharing of class I HLA antigens (Oksenberg et al. 1983, Mowbray et al. 1983, Johnson et al. 1984) but rather, a depressed cellular response between the aborting mother and her husband's cells but not other partners (Laruitsen et al. 1976). Mowbray's group has shown that although class I sharing was not higher than expected, statistically significant class II antigen sharing occurred (Mowbray and Underwood 1985). This concurs with Ober's observations on the HLA sharing among Hutterites. She found that HLA DR sharing results in decreased fertility in such couples (Ober et al. 1987).

Other studies indicate that in rodents (Chaouat et al. 1983, 1985) and horses (Antczak and Allen 1984), immunization of the mother with cells of a histocompatibility type related to the paternal MHC can in some cases promote fetal survival. As I mentioned earlier, Clark and his associates observed that CBA/J murine females pregnant by DBA/2J males show an increased rate of spontaneous abortion when compared to other strains or to the reciprocal strain combination (Clark et al. 1984). Immunization of the CBA/J females with BALB/cJ (but not DBA/2J, BALB.B, BALB.K or CBA/J) spleen cells lowered the resorption rate from 25% to 5% (Chaouat et al. 1983, Kiger et al. 1985) and increased the active suppression against NK cells in the placenta. Successful vaccination correlates with the appearance of anti-paternal MHC antibodies in the maternal serum which may passively transfer protection to naive virgin CBA/J females (Chaouat et al. 1985).

Antczak and Allen (1984) have noticed that the transfer of donkey embryos to horse foster mothers results in failure of endometrial cup formation, and only 10% of these pregnancies go to completion. However, transfer of serum from normal horse fetuses or immunization with paternal lymphocytes prior to donkey transfer can result in the birth of normal donkey offspring in 90% of the cases.

C) OBJECTIVE AND RATIONALE

These observations point into two different directions. One could speculate that a portion of the maternal immune response generated during pregnancy accounts for the generation of suppressor activity, whereas another portion accounts for the generation of growth factors stimulating placental and fetal growth. Indeed, it was shown that the same cell population in the decidua secretes a suppressor factor (Clark et al. 1984), in addition to a factor(s), stimulating placental cell proliferation and phagocytosis (Athanassakis and Wegmann 1986). Since the number of T cells, which are known to secrete many growth factors, increases during pregnancy, these cells are the best candidates for the stimulatory activity exerted on the placenta and fetus. Therefore, the present study attempted to provide a link between maternal T cells and successful pregnancy in the context of maternal immune stimulation and not immune suppression. Although T cells could not be detected in the placenta *per se* (chapter II), they could exert their stimulatory effect through their products in the vicinity of the placenta. This thesis has as its focus the postulate that once maternal T cells become activated by fetal alloantigens, they can trigger placental proliferation through lymphokine release and thus improve the probability of fetal survival. Indeed we were able to show that T cell-derived multipotential colony-stimulating factors, Interleukin-3 (IL-3/multi-CSF) and the granulocyte-macrophage CSF (GM-CSF), as well as the colony stimulating factor-1 (CSF-1) can stimulate proliferation of a placental cell population which is of fetal origin. These cells are adherent, phagocytic, stain positively for non-specific esterase and bear CSF-1 receptors. Further analysis indicated that these cells are trophoblasts, which also express markers in common with monocytes. The inability of these "trophoblast-macrophages" to proliferate in response to growth factors when separated from the smaller cell population of fetal macrophages in the placenta suggests the presence of a regulatory network between maternal T cells, trophoblasts and macrophages. Parallel experiments *in vivo* show that ablation of maternal T cells with specific antisera decreases placental cell proliferation and phagocytosis. Finally we showed that decidual cell supernatants contain growth factors which promote placental cell growth. This activity was higher in decidual supernatants from allogeneic than from syngeneic pregnancies, and it was significantly reduced when the females were previously treated with anti-T cell monoclonal antibodies. Most of the activity in the decidual supernatants appears to be GM-CSF-derived, since it is blocked by anti-GM-CSF antibody. The next three sections represent manuscripts that describe this work in detail.

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CHAPTER II

The Immunostimulatory Effect of T cells and T cell lymphokines on murine fetally - derived placental cells.[1]

INTRODUCTION

The mammalian placenta serves as an immunological barrier between the maternal and fetal circulations, preventing the potentially destructive maternal immune response from damaging the semiallogeneic fetus (1). The manner in which this barrier functions is the subject of intense current investigation (2). One hypothesis advanced to explain fetal survival is that local immunosuppression prevents the sensitization of the maternal immune system to paternal alloantigens and development of subsequent effector functions (3-6). A related possibility is that maternal immune recognition of fetally-derived cell surface antigens results in the secretion of factors that promote the growth of placental tissue, which in turn provides a fortified barrier to external damage from maternal immunity or microbes (7). This mechanism, termed the immunostimulation hypothesis, has direct analogy to experimental situations in which tumor cells are stimulated rather than inhibited by an immune response (8,9). This, of course, could coexist with immunosuppression so that the local maternal immune response does not destroy the pregnancy. Indirect experimental support for the immunostimulation hypothesis came from early observations that F1 hybrid murine placentae are larger than those of either inbred paternal strain (10). It has also been reported that females immune to paternal antigens produce larger placentae than tolerant females (11,12). Based on these observations, some authors have suggested that the maternal immune response is necessary for, or at least has a beneficial effect on, the maintenance of pregnancy (13-15). However, the evidence in support of this idea is weak and subject to strain variation (16). Recent observations with respect to spontaneous abortions in humans have renewed interest in the immunostimulation hypothesis. Women undergoing multiple spontaneous abortions with no obvious chromosomal, endocrinological or gynecological abnormalities have been observed to carry pregnancy to term if first immunized with paternal or third-party leukocytes (17-20). Other studies indicate that in rodents (21,22) and horses (23) immunization of the mother with cells related to the paternal major histocompatibility complex can in some cases promote fetal survival. A direct molecular explanation for these maternal immune effects on fetal survival however, is lacking. Morover,it is reasonable to think that immunoregulation affects the placenta, where maternal immune cells come into direct contact with fetal tissues.

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[1] This chapter has been published. Irene Athanassakis, R. Chris Bleackley, Verner Paetkau, Larry Guilbert, Philip J. Barr, Thomas G. Wegmann. Journal of Immunology. 138: 37-44: 1987.

In this paper we describe *in vitro* experiments which indicate that CSF-GM and IL-3, generally accepted as T cell-derived lymphokines, provide a possible molecular basis for the immunostimulatory effect observed on the placenta *in vivo*. We demonstrate that the placental cells which proliferate in response to CSF-GM are fetal in origin, and appear to be macrophage-like. Finally we show that elimination of maternal T cells during pregnancy by *in vivo* injection of either anti-thymocyte serum or anti Ly2.1 monoclonal antibody leads to a reduction in the proliferative and phagocytic capacities of the day 12 placentae. These observations support the hypothesis that maternal T cells can stimulate placental proliferation through lymphokine release, thereby improving the probability of fetal survival.

MATERIALS AND METHODS

Mice

BALB/cJ and C3H/HeJ were obtained from the Laboratory Animal Breeding Unit at the University of Alberta (Ellerslie, Alberta) or the Jackson Laboratories (Bar Harbor, ME). BALB/cJ and C3H/HeJ mice differ at the glucose phosphate isomerase locus (Gpi-1); C3H/HeJ are homozygous for the fast electrophoretic variant Gpi-1 /Gpi-1 while BALB/cJ are homozygous for the slow variant, Gpi-1 /Gpi-1 . Each BALB/cJ or C3H/HeJ female was checked for oestrus, caged overnight with a BALB/cJ male and examined for the presence of a vaginal plug the following morning. The day on which the plug was observed was considered to be day zero of pregnancy.

Placental cell cultures

Placentae were obtained from females on day twelve of pregnancy. After removing the maternally-derived decidual layer(24), which in our hands carried the percentage of fetal glucose phosphate isomerase isozyme in the placenta from 44% to 55% (Athanassakis I. unpublished observations), single cell suspensions were prepared by pushing the placenta through a ~~fine mesh wire screen~~ into Leibowitz medium (Gibco, Grand Island, NY). The cells were washed three times and cultured in RPMI 1640 (Gibco) supplemented with 10% FCS (Flow Lab, Mississauga, Ont) at 10^7 cells/ml in 96 well plates (Linbro, Flow Labs McLean, Virginia) with or without various lymphokines and mitogens. Mitogens used for activation were either Concanavalin A (Con A, 2.5 μg/ml) or phorbol myristate acetate (PMA, 10 ng/ml) and were always added at the beginning of culture. Cultures were assayed for ^{3}H Thymidine (^{3}HTdR) incorporation on days two or four. One μCi of ^{3}HTdR was added per well 18 hours prior to harvest, and the cells were placed in scintillation fluid (toluene/omnifluor - NEN, Boston,MA, 1.38 g/l) and counted for beta emission in an LKB 1218 Rackbeta Counter.

Lymphokines

Partially purified lymphokines were isolated by chromatography of culture supernatants of the T-cell derived lymphoma EL4 (variant E1) which had been activated with PMA (10 ng/ml). This preparation, termed F3, contains at least the following lymphokines: IL-2, IL-3, CSF-GM, BCGF and B-cell differentiating factor (BCDF)(25). Further purificaton was achieved using gel filtration columns, yielding the following fractions : fraction 4' (effluent of a Phenyl Sepharose column) containing mainly CSF-GM, fraction 5'(effluent of a DEAE-Sephacel column) which is largely IL-3, and fraction 6 (obtained by reverse-phase

high performance liquid chromatography) which is pure IL-2 (25). Partially purified CSF-1 was obtained from serum free L-cell conditioned medium (27).
Recombinant murine CSF-GM (rCSF-GM) was produced in yeast from synthetic genes essentially as described by C.R. Bleackley et al.(26) (a complete account of this yeast expression system will be described elsewhere).
Recombinant IL-3 expressed in the COS (monkey) cell line was a generous gift of Dr. Timothy Mossman (DNAX Corporation, Palo Alto, Ca)(28). The activity of the IL-3 preparation was tested on the IL-3 dependent cell line DA-1 (data not shown).

Fractionation of placental cells

Three day old BALB/cJ blastocysts were surgically transfered to the uteri of C3H/HeJ females in their second day of pseudopregnancy as determined by vaginal plug formation after mating with vasectomized males. Single cell suspensions from twelve day placentae were obtained as described above, and then centrifuged at 2000 rpm for 30 min through a discontinuous Percoll gradient (80% - 65%, Pharmacia, Dorval, P.Q.). The cells collected at the 80-65% interface, which are of maternal origin by GPI typing (Athanassakis I. unpublished observations), were discarded. The remaining cells (67% fetal by GPI typing) were washed and cultured with or without monoclonal αH2-K^k

antibody, rabbit complement (Low-Tox at a dilution of 1:6 v/v, Cedarlane, Hornby, ONT) and CSF-GM. The αH2-K^k antibody is a protein-A purified IgG fraction derived from ascites fluid produced by the cell line 16-3-1N (29), and was used at a dilution of 1/100 (v/v). Control experiments indicate that under these conditions it is cytotoxic for C3H/HeJ (H-2K^k) but not BALB/cJ (H-2K^d) spleen cells. On day four of culture the cells were assessed for proliferation by ^{3}HTdR incorporation and maternal versus fetal origin. The latter was assessed by electrophoretically determining the isozyme of glucose phosphate isomerase as described elsewhere (30). Briefly the cultured cells were collected, washed in PBS, submerged in liquid nitrogen and stored at -70°C until electrophoresis was performed. GPI isozymes in the cell lysates were separated by using starch gel electrophoresis and were developed by the nitrocellulose overlay technique (30,31). Quantitative measurements of the relative contributions of maternal and fetal cells was determined by scanning the stained nitrocellulose filters (mounted on microscope slides) in an Electrophoresis Scanner model 80100 (Camag, Mutlenz, Switzerland).The signal produced was analysed by using a Hewlett-Packard mode 3390A Integrator (Palo Alto, CA) which determined the area of each band as a percentage of the total pattern.

Placental Cell Phagocytosis in vitro

Placental cells were cultured in the presence of various lymphokines in 96 well plates. After four days 5μl of fluorescent beads (fluoresbate fluorescent monodisperse carboxylated microspheres, D=0.57μ, Polysciences Inc, Warrington, PA) were added per well and the cultures were incubated for 30 minutes at 37°C. After washing the adherent layer 5 times, 0.2ml of PBS were added per well and phagocytosis of the beads determined by measuring absorbance at 414 nm in an ELISA plate reader (Titertek Multiskan). The background (optical density of cells prior to phagocytosis) was substracted from the values obtained. In another series of experiments bacto-latex beads (Difco) were used and phagocytosis was evaluated by microscopic inspection.

Non-Specific-Esterase Staining

The placental cells were cultured in chamber slides (Lab-Tec Miles Lab, Naperville, IL) under the conditions described above. The adherent layer was first assessed for phagocytosis of fluorescent beads and then stained for non-specific esterase according to the method described by I. R. Koshi et al (32).

Autoradiography

One µCi of ^{3}HTdR was added to each well containing placental cells on day 3 of culture. On day 4 cytospins of the adherent and non adherent layer were obtained, fixed in ice-cold methanol for 20 minutes, rinsed in distilled water and air dried. The slides were dipped in warm (42°C) emulsion (Kodak NTB2,Eastman Kodak Co.,Rochester, N.Y.), dried and enclosed in light-excluding containers for 4 days at 4°C. After warming to 22°C the slides were developed for 2 minutes in Kodak D-10 developer,rinsed for 30 seconds in 1% (v/v) acetic acid, fixed for 3 minutes in Kodak fixer and washed in 5 changes of distilled water. After drying, the preparations were stained with Giemsa at pH 6.5, and the percentage of cells containing nucleus confluent with silver grains was estimated.

T cell depletion in vivo

C3H/HeJ females pregnant by BALB/cJ males were injected three times intraperitoneally (i.p.) with 0.1ml of rabbit anti-mouse thymocyte serum (ATS, M.A. Bioproducts, Walkersville, MD, 98% killing of thymocytes at 1:80 dilution *in vitro*), on days 8,10 and 11 of pregnancy. This treatment was found to give maximal suppression of the splenocyte proliferative response to phytohemaglutinin (PHA). ATS suppression was measured as follows : 1×10^7 spleen cells/ml were cultured in RPMI medium (Gibco Lab, Grand Island, NY) supplemented with 10% Fetal Calf Serum (FCS,Flow) with or without PHA (1/40 dilution) and assessed for proliferation on day four of culture by ^{3}HTdR uptake. A similar protocol was used for treatment with the anti-Ly2.1 (HB129, ATCC, Rockville, Maryland) monoclonal antibody (33). Placental proliferation *in vivo* was assessed by injecting the mice with 27μCi of ^{3}HTdR i.p.. The following day the mice were killed by cervical dislocation and all placentae and spleens were removed. Individual placentae were cut into small pieces using fine scissors, put in 3ml of NCS-ACS solution (1:10, Tissue solubilizer/aqueous counting scintillant, Amersham, Oakville, ONT) , shaken thoroughly and counted in an LKB beta counter. Each spleen was individually tested for responsiveness to PHA.

In other experiments the mice were treated with normal rabbit sera (NRS) or ATS absorbed with T cells (anti Poly 18.68 T helper cell line, ref. 34) as controls. The ATS was absorbed by incubating the packed T cells with an equal volume of ATS for 45 minutes at 37°C.

Placental Cell Phagocytosis in vivo

Cell suspensions of individual placentae from day 12 pregnant mice were washed and incubated with fluorescent beads as above. The cells were then washed and red cells lyzed by hypotonic shock. After two washes the cells were resuspended in PBS and optical density (OD) determined in an ELISA Counter. In some experiments a visual assessment of phagocytosis using a fluorescent microscope was also established.

In another series of experiments the fluorescienated latex beads were injected directly into the uterine artery of anesthetized females and 30 min later the placentae were either dissociated and determined in the ELISA reader or processed for histological examination. For the latter, placentae were isolated, fixed in 10% formalin, dehydrated, paraffin embedded and longitudinally sectioned. Sections were then stained with Hematoxalin and Eosin and microscopically examined using brightfield and fluorescent light.

RESULTS

T Cell-derived Lymphokines Stimulate Placental Cell Proliferation

As an initial test of the immunostimulation hypothesis, we examined whether cells derived from 12 day placentae can proliferate in response to various T cell-derived lymphokines. Placental cells were cultured for 48 hours with a partly purified preparation (F3, 1/40 v/v) of medium conditioned by the T-cell-derived lymphoma EL-4 (variant E1), which had been activated with PMA (25). F3 produced an eleven-fold increase in ^{3}H-thymidine incorporation by placental cells compared to these cells cultured in the absence of EL-4 supernatant (Table 1). To determine which lymphokine gives optimal proliferation, fractionated samples of F3 including CSF-GM, IL-3 or IL-2 were added in graded doses to placental cell cultures. A significant increase in ^{3}H-thymidine incorporation was observed with the CSF-GM preparation. The stimulatory effect was not due to activation by residual PMA, since PMA was unable to augment placental cell proliferation in the presence or absence of the lymphokines (data not shown). The CSF-GM preparation may sometimes contain BCDF activity; however, the fraction that was used is free of BCDF since it was unable to stimulate B cell proliferation and antibody production. Likewise, recombinant CSF-GM stimulated significant proliferation (Table II). Pure IL-2 was unable to stimulate placental cell proliferation even at high concentations. Although rIL-3 increased the ^{3}HTdR incorporation in a dose dependent manner, column purified material had little effect (Table II) probably due to the low activity of the latter preparation (added at 0.3 IU/ml as compared to 6 IU/ml rIL-3 needed for maximal proliferation).

Lymphokines are known to be short-lived and some cells require more than one lymphokine to proliferate. To evaluate whether placental cells exhibit these properties, the following experiments were done. We first showed that two doses of CSF-GM, given at initiation and at 48 hours of culture, almost doubled the stimulatory effect obtained with a single addition at the beginning of culture. The interval of 48 hours between the two additions of CSF-GM was necessary in order to increase proliferation, since a double dose added either at the onset, 18 or 24 hours of culture gave no greater stimulation than that obtained by a single addition of CSF-GM at the onset. Furthermore, CSF-GM given at initiation of culture and followed 48 hours later by IL-3 increased the proliferation 13 fold. The reverse combination, IL-3 followed by CSF-GM at 48 hours, did not increase proliferation over that caused by

IL-3 alone. The colony stimulating factor-1 (CSF-1), which stimulates only macrophage production (35), was able to activate placental cells and allow them to reach the same level of proliferation as that seen with the partially purified CSF-GM (Table II).

Lymphokine-Responsive Cells in the Placenta are of Fetal Origin

The 12 day placenta is a chimeric organ which contains an approximate one to one ratio of fetal to maternal cells when made into cellular suspension (24). It is therefore necessary to examine the origin of the proliferating cells in the placental cell cultures. In order to distinguish between maternal and fetal cells in the placenta, we typed the cells for GPI isozyme expression by microstarch gel electrophoresis. Mice that are homozygous for the *Gpi-1* allele express an isozyme that migrates faster in an electric field than the form expressed by *Gpi-1* homozygous mice. Heterozygotes express both bands as well as a heterodimeric band with intermediate mobility. Therefore a mixture of homozygous and heterozygous cells does not allow easy quantitation because of band overlap. To circumvent this problem, we transfered BALB/c X BALB/c preimplantation blastocysts (*Gpi-1*) into the uteri of pseudopregnant C3H/HeJ (*Gpi-1*) females. The fetal and maternal GPI bands are thus electrophoretically unambiguous and lend themselves to sensitive quantitation using densitometry techniques. The isolated placental cells were incubated with various combinations of anti-maternal class I monoclonal antibody (anti H-$2K^k$), complement, and CSF-GM (added at the beginning and at 48 hours of culture), and assayed for proliferation and isozyme content. The results, shown in Table III, clearly indicate that the cells which proliferate in response to CSF-GM must be fetally derived, because virtually complete elimination of the maternal cells, as shown by isozyme analysis,does not quantitavely affect CSF-GM-driven proliferation.

The Proliferating Placental Cells Display Macrophage or Trophoblast Characteristics.

The placental cells proliferating in response to CSF-GM were examined for their microscopic appearance after four days in culture. Two populations could be distinguished: non-adherent and adherent. The non-adherent cells displayed variable morphology including both maternal and fetal red blood cells and an irregularly shaped granular cell of undefined origin. The adherent layer contained primarily large, granular, vaculated cells. Autoradiographic studies showed that only the adherent cells proliferated in response to CSF-GM, since 34±8% of these cells (which represent 12% of the total cell population) incorporated ^{3}HTdR at day four of culture. This contrasts with the non-adherent cells, where only 0.5± 0.3% (0.3% of total cells) proliferated (Table IV).

Since the adherent cells are proliferating in response to lymphokines that are known to stimulate macrophages, and since they morphologically resemble macrophages, we next inquired whether they display other macrophage characteristics. Thus we stimulated these cells with CSF-GM or IL-3 and then assessed their phagocytic capacity on day four of culture by measuring the uptake of fluorescent beads as determined by optical density readings. Parallel cultures were assessed for ^{3}HTdR incorporation. There was a direct correlation between the amount of proliferation and phagocytosis (r=0.99) (Figure 1A). Phagocytosis was further monitored by microscopic examination of adherent cells stimulated by CSF-GM or IL-3 and then incubated with bacto-latex beads. Cells proliferating in response to CSF-GM are large and have a darkened cytoplasm, due to the incorporation of the latex beads. The adherent cells in the IL-3 stimulated cultures are considerably smaller and do not have the darkened cytoplasm characteristic of extensive bead uptake (Figure

1B). In another set of experiments the cells were incubated with fluorescent beads and subsequently stained for non-specific esterase. All phagocytic cells stained positively for esterase whereas non-phagocytic cells were negative (Fig. 1C). Fewer than 5% of adherent cells growing in response to CSF-GM were found to bear Mac-1, a plasma membrane antigen typical for murine macrophages (36)(data not shown). Thus the fetally-derived placental cells that proliferate in response to CSF-GM are adherent, phagocytic esterase positive and Mac-1 negative.

Maternal Treatment with ATS Reduces Placental Proliferation in vivo

Having demonstrated that T cell lymphokines enhance fetally-derived placental cell proliferation *in vitro*, we next inquired whether proliferation and phagocytosis in the intact placenta could be reduced by maternal T cell depletion, as would be predicted by the immunostimulation hypothesis and the results presented above. This was first addressed by giving three i.p. injections of 100µl of ATS on days 8, 10 and 11 of pregnancy. This treatment consistently suppressed the PHA-induced proliferation of splenocytes to approximately 5% of normal levels. On day 11 the animals were further injected with 27 µCi of ^{3}HTdR i.p. and 24 hours later individual placentae were removed, processed and assessed for ^{3}HTdR uptake. The ATS -treated females showed approximately 50% reduction in the placental proliferation as compared to that seen in non-treated or NRS-treated pregnant mice (Figure 2). All 17 females displaying reduced PHA responsiveness in their splenocytes also had reduced placental ^{3}HTdR incorporation. Three females that had no reduction in their splenocyte response to PHA also showed no reduction in placental ^{3}HTdR incorporation (data not shown).

The data in Figure 2 also indicate that the ATS treatment had a slight but statistically insignificant effect on fetal resorption. There was no difference in placental weight between the ATS and non treated groups (29.9±2.4 mg versus 30.5±1.6 mg, respectively).

To strengthen the conclusion that the ATS-mediated reduction of placental proliferation is due to its reactivity against maternal T cells and not to non-specific killing of another maternal cell population, we absorbed the ATS with an equal volume of cloned T helper cells. This pretreatment eliminated the ability of ATS to reduce placental ^{3}HTdR incorporation *in vivo* (Figure 2). Curiously, although the absorbed serum did not affect placental cell proliferation, splenocytes from these mice still showed reduced PHA responsiveness (Figure 2). This could indicate that a non T cell contributes to PHA responsiveness and is eliminated by the ATS treatment.

In view of the above results, two other explanations for the observed effect of ATS on placental proliferation *in vivo* were considered. The first is that mature T cells in the placenta are responsible for the reduction of ³HTdR uptake. To determine whether functional maternal T cells exist in the day 12 placentae, a placental cell preparation was cultured for 4 days with 2.5μg/ml of Con A. This treatment did not significantly increase ³HTdR incorporation above background levels (1,123±160 versus 1,001±225 cpm in control cultures).

A second possibility is that ATS has a direct cytotoxic effect on fetally-derived placental cells. To address this question, we determined whether ATS and complement could abolish the *in vitro* proliferation of placental cells in response to CSF-GM. Placental cells from C3H/HeJ X BALB/cJ pregnancies were cultured with or without CSF-GM, ATS and complement. The presence of ATS and complement in the culture did not affect the proliferative activity of placental cells in response to CSF-GM, while completely eliminating the PHA responsiveness of parallel cultures of spleen cells (Table V).

Maternal Treament with ATS Reduces Placental Phagocytosis in vitro and in vivo

Since CSF-GM caused placental cells to develop phagocytic function *in vitro*, the results in Figure 2 predict that ATS treatment *in vivo* would reduce placental phagocytic capacity . We tested this possibility in two ways. In the first set of experiments, day 12 placentae from ATS-treated mice were made into cell suspensions and 10^5 cells incubated for thirty minutes with 5µl of fluorescent beads. Phagocytosis was then determined in the ELISA plate reader. The results indicate that placentae from ATS treated mice showed half as much phagocytic capacity as control placentae. Microscopic examination of the same cell preparation indicated a much lower percentage of phagocytic cells in ATS-treated versus normal placentae (Table VI). A second method of examining the reduction in phagocytosis was to directly inject 100µl of the beads into the lumen of the uterine artery of ATS-treated and control animals. Placental cell preparations could then be examined directly in the ELISA plate reader. The results indicated a significant reduction in phagocytosis of the ATS treated placentae . Finally, to identify the phagocytic cells in the placentae injected with fluorescent beads, hematoxalin and eosin stained sections were prepared. Virtually all the cells containing fluorescent beads were large mononuclear cells located in the compact region of the labyrinthine zone (Figure 3). These cells are ordinarily described as trophoblast cells by morphological criteria .

Maternal Treatment with anti-Ly2.1 Reduces Placental Proliferation and Phagocytosis in vivo

Because of the heterogeneous nature of the antiserum preparations, the results obtained with ATS do not eliminate the possibility that the ATS is affecting some fetal cell population that influences placental proliferation and phagocytosis. We therefore applied the same injection protocol using mouse anti-Ly2.1 monoclonal antibody which more specifically depletes pregnant females of Ly2 positive T cells. Table VII shows that the treatment causes a reduction in both placental proliferation and phagocytosis comparable to that seen with ATS. Since the Ly2 marker does not appear until after day 14 of ontogeny (37, 38), two days after the termination of the experiment, these results also demonstrate that maternal and not fetal T cells contribute to placental proliferation and phagocytosis.

DISCUSSION

The experiments reported here were designed to test two direct predictions of the immunostimulation of placental growth hypothesis. The first is that T-cell derived lymphokines should be capable of stimulating growth and/or activation of fetally-derived placental cells *in vitro*; the second is that maternal T cell ablation during pregnancy should retard placental cell proliferation and function *in vivo*. The results are in accordance with these predictions.

The first observation was that placental cell preparations, containing both maternal and fetally derived cells, would proliferate in response to crude supernatants of PMA-stimulated EL-4 cells. It is important to note that neither PMA nor Con A stimulated placental cells to proliferate, indicating that they are not significantly contaminated with maternal T cells. By using partially purified as well as recombinant lymphokines virtually the entire proliferative effect could be ascribed to lymphokines of the CSF family, including CSF-GM, CSF-1 and IL-3. IL-2 and BCDF had no significant effect either alone or in conjuction with CSF-GM. CSF-GM, IL-3 and CSF-1 preparations are known to induce proliferation of murine cells of the macrophage lineage (35,39). The existence of growth factors specific for macrophage subsets has been suggested (35) but not yet been described.

Having identified which lymphokines could stimulate placental cell proliferation we then proceeded through a series of steps to characterize these cells. The first point established was that virtually all of them are of fetal origin. To do this, we implanted embryos that were homozygous for both the MHC and the GPI isozyme locus into pseudopregnant females homozygous for different alleles at these loci. This allowed us to show that although monoclonal anti-maternal MHC class I antibody plus complement virtually eliminated cells bearing maternal isozyme from CSF-GM-stimulated cultures, it had no effect on proliferation. Thus it is very likely that the cells in question are of fetal origin.

The placental cells that incorporate ^{3}HTdR were large, adherent to plastic and positive for non-specific esterase staining, suggesting their macrophage-like nature. In addition, these cells proliferate in response to the same growth factors as do macrophages; ie, CSF-1, thought to be specific to mononuclear phagocytes (40, 41), CSF-GM and IL-3 (35). Although the cells are Mac-1 negative, some (Kupffer cells) macrophages lack this antigen (36). Nonetheless, the physical properties of the lymphokine-responsive cells describe both trophoblasts

(42,43) and macrophages. Therefore, the cells are being further characterized by a panel of monoclonal antibodies that will more clearly distinguish between the two possibilities. Identification of the cell as macrophages would point to a central role for these cells in placental growth. If, on the other hand the cells are trophoblasts, they would have to share the growth factor responsiveness of macrophages. Interestingly, transcript to the proto-oncogene *c-fms*, which encodes the receptor to the macrophage-specific factor, CSF-1 (44), has been identified in the human trophoblastic cell line BeWO (45). Thus, it is possible that trophoblasts either are macrophages or have evolved identical physical properties and growth requirements.

Either of the above possibilities is in accord with our suggestion that lymphokines stimulate placental growth. Other investigators have identified macrophage-like cells in both human (46,47) and murine (48) placentae, but their role as well as their relation to the trophoblast lineage, is still unclear. One possibility is that these cells filter out harmful anti-fetal antibodies, via their Fc receptors, during passsage of immunoglobulin from the mother to the fetus (49-52). Another is that they provide a phagocytic barrier to the entry of harmful microbes and maternal cells into the fetus.

The second series of experiments were designed to test whether immunostimulation of the placenta by maternal T cells occurs during normal pregnancy . To evaluate this, we treated pregnant females with rabbit anti-mouse thymocyte serum. This treatment lowered both the proliferative and phagocytic capacity of intact 12 day placentae to half their normal values. Since a heterologous antiserum was utilized, it was necessary to ascertain that the effect was due to ablation of maternal T cells and not to a direct cytotoxic effect on fetal or placental cells. The former conclusion was strengthened by absorbing the ATS with cloned T cells, which eliminated the effect of the ATS treatment on the placenta. In addition ATS plus complement, which is effective in eliminating PHA responsiveness *in vitro*, had no effect on placental cell response to CSF-GM. Nevertheless, the ATS could be affecting a fetal cell population producing placental growth factors. To rule this out, we treated the pregnant females with monoclonal anti-Ly2.1 which reacts with T cell subsets that are not present in the 8-12 day old fetus. Comparable results were obtained, clearly implicating maternal T cells in the placental proliferation and phagocytosis.

These results, then, conform to one prediction of the immunostimulation hypothesis, namely that maternal T cell elimination should have a measurable effect on placental proliferation and function. This conclusion is strengthened by comparing the *in vitro* results to those found *in vivo*. In each case there is a close correlation between the amount of placental cell proliferation and phagocytosis, whether both are enhanced by CSF-GM *in vitro*, or reduced by ATS treatment *in vivo*.

Experiments involving injection of fluorescent beads in the uterine artery permit a tentative identification of the cells involved in phagocytosis, based on subsequent placental histology. Virtually all the fluorescence was found in the trophoblast cells of the compact area of the labyrinthine zone. Other workers have also determined that these are the principle phagocytic cells in the placenta. In addition, we have shown, using the GPI isozyme analysis, that the labyrinthine zone has no detectable maternal cell contribution, strengthening our conclusion that the cells in question are fetally-derived (Athanassakis et al, manuscript in preparation).

In conclusion, these experiments lend credence to the idea that maternal T cells contribute to the growth and functional capacity of the placenta by lymphokine stimulation. The function in question is phagocytosis, which has an obvious relation to fetal health and viability. The experiments of Edidin and his colleagues are relevant in this context (8) . These investigators showed that peritoneal exudate cells from multiparous but not virgin female mice can induce proliferation of both teratocarcinoma cells and embryonic blastocysts *in vitro*. A number of groups have shown that cells in the vicinity of the fetoplacental unit are nonspecifically immunosuppressive and release factors that mediate immunosuppression of maternal lymphocytes *in vitro*. It is possible that the true *in vivo* function of these cells is to provide growth factors for the placenta, but the two possibilities are not mutually exclusive, and may coexist to provide placental growth in the absence of immune damage. Since neither treatment with anti-thymocyte serum nor anti-Ly2.1 monoclonal antibody significantly compromize fetal viability, one cannot claim that maternal T cells are essential for fetal survival under conditions of optimal animal care. It is clearly well known that nude mice can breed under appropriate germ-free conditions. The significance of the current observations may pertain to conditions in which pregnancy is compromized by microbial assault. Nevertheless, the current experiments forge a more direct link between maternal immune function and placental differentiation, although the details of this interaction await better characterization of the cells, antigens and lymphokines involved.

Acknowlegments

We thank Dr. L. Honore for useful discussions on the histology of the placenta. We also thank Rose Hunziker and Michael Voralia for help with the manuscript and Ann Semeluk,Cliff Gibbs, Donald Macrae, Elizabeth Sabin for technical assistance.

Fig. 1. Corrrelation between proliferation and phagocytosis of placental cells in culture. BALB/cJ x BALB/cJ placental cell suspensions were cultured for four days as follows : placental cells alone as a negative control (I), placental cells treated with IL3 at the onset of culture (II), placental cells treated with CSF-GM at the onset of culture (III) and placental cells treated with CSF-GM at the onset and at 48 hours of culture (IV).

1A shows the correlation (r=0.99) between proliferation and phagocytosis of the adherent layer of these cells after 4 days

1B shows the adherent layer of the two groups II and IV phagocytosing bacto latex beads. Magnification x40.

1C shows that the phagocytic cells are also esterase positive.

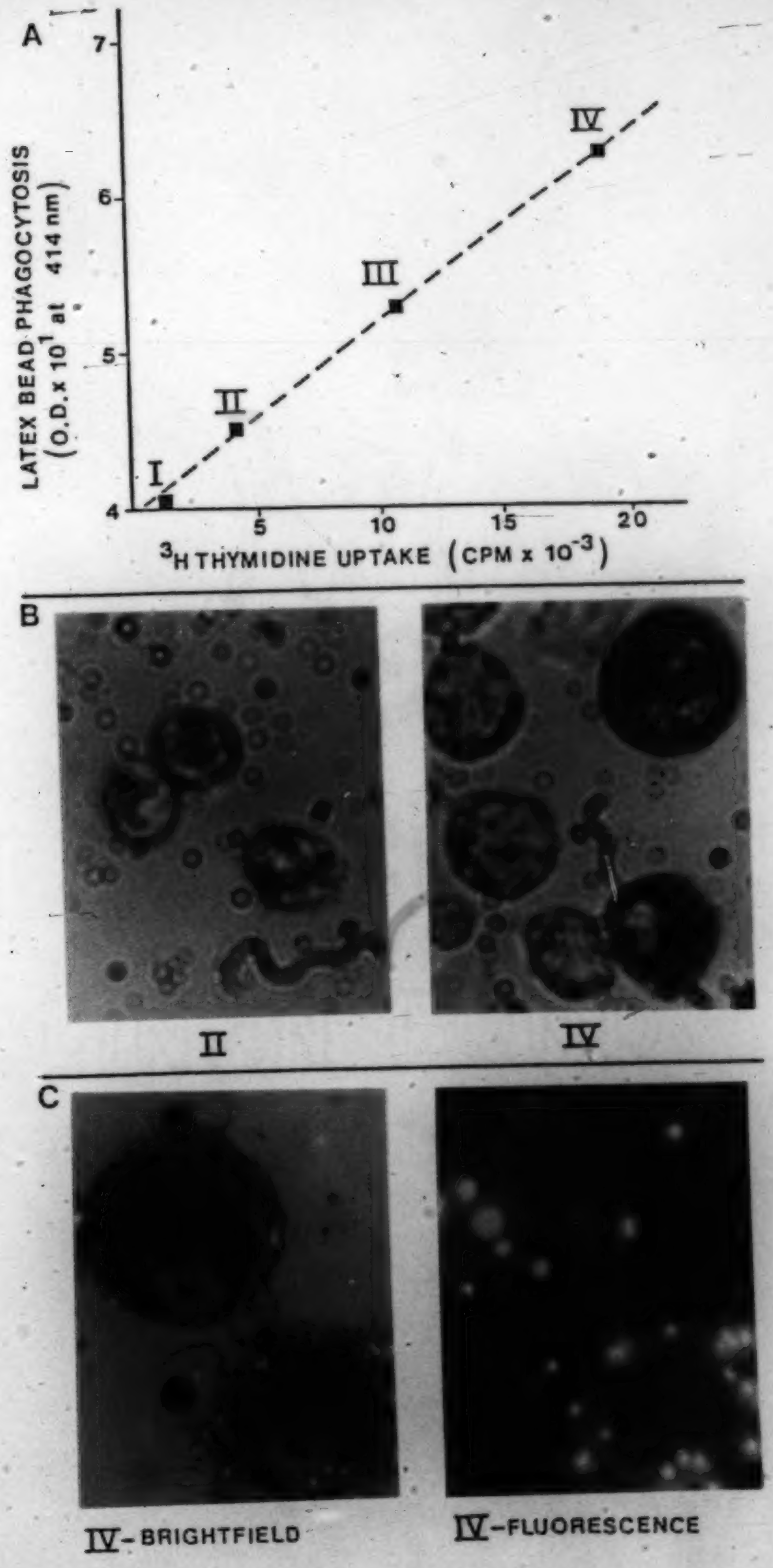
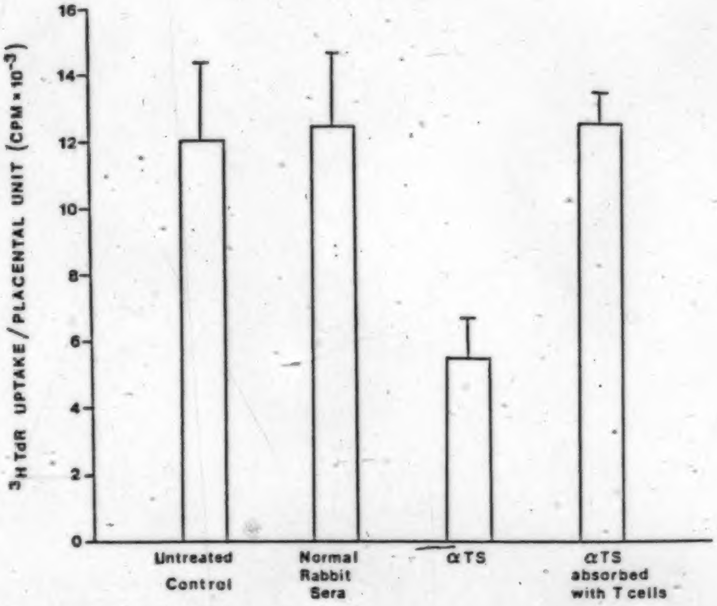

A
LATEX BEAD PHAGOCYTOSIS (O.D. x 10^1 at 414 nm)
4
5
6
7
^{3}H THYMIDINE UPTAKE (CPM x 10^{-3})
5
10
15
20
I
II
III
IV
B
II
IV
C
IV-BRIGHTFIELD
IV-FLUORESCENCE

Fig. 2. ATS treatment reduces placental proliferation *in vivo*. C3H/HeJ females pregnant by BALB/cJ males were immunosuppressed with ATS and injected with ^{3}HTdR in order to test proliferation. Individual placentae (placental unit) were isolated and the whole tissue was counted after dissociation in NCS-ACS solution (see materials and methods).



# of Placentae shown above	67	81	108	16
# of Resorbed Embryos	10	17	29	4
% of Normal Embryos	89±11	85±10	73±7	75±4
# of Mice	13	13	17	3
% of Suppression in spleen in response to PHA	0	0	94±3	97±4

Fig. 3. Localization of phagocytic cells in the placenta. C3H/HeJ females pregnant by BALB/cJ males were injected on day 12 of pregnancy in the uterine artery with 100μl of fluorescent beads. Thirty minutes later the placentae were removed, fixed in 10% formalin, paraffin embedded and longitidinally sectioned. The sections were then stained with hematoxilin and eosin and photographed under brightfield (A) and fluorescent (B) light (x40x3.3). LB:labyrinthine trophoblast, SP:spongiotrophoblast. Arrows indicate examples of trophoblast cells which are phagocytic and become fluorescent after ingestion of fluorescent beads. The same field was photographed under normal and fluorescent light (x40x3.3). Only cells from group IV are shown.

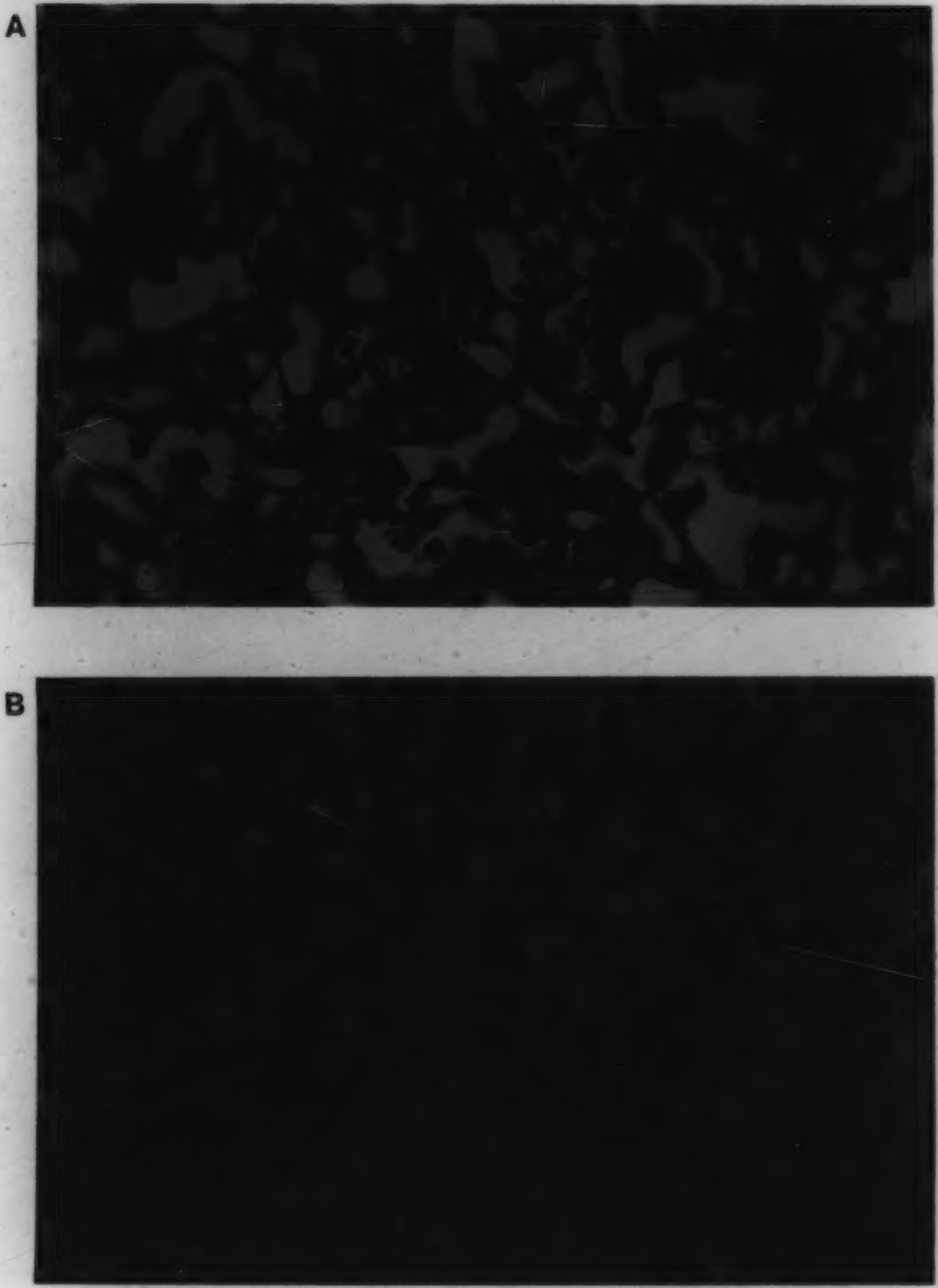

A
B

TABLE I

EFFECT OF VARIOUS LYMPHOKINES ON PLACENTAL CELL PROLIFERATION IN VITRO

Group Number	T-cell lymphokine at beginning of culture	T-cell lymphokine at 48 hours of culture	Day of culture harvest	^{3}H TdR Incorporation* (cpm ± SD)
1	—	--	2	1,001 ± 225
2	F3	--	2	11,518 ± 1,856
3	CSF-GM	—	2	10,962 ± 524
4	IL3	—	2	4,137 ± 691
5	IL2	—	2	3,729 ± 241
6	—	—	4	1,078 ± 241
7	CSF-GM	--	4	11,825 ± 1,091
8	CSF-GM	CSF-GM	4	18,889 ± 1,880
9	IL3	IL3	4	3,189 ± 366
10	CSF-GM	IL3	4	13,343 ± 1,769
11	IL3	CSF-GM	4	4,372 ± 450

* 1 uCi/culture, added 18 hours prior to harvest

TABLE II

Recombinant CSF-GM and IL-3 stimulate placental cell proliferation the same way as purified CSF-GM, IL-3 and CSF-1.

	^{3}HTdR uptake (cpm±SD)	
	Recombinant factors	Column purified factors
Exp. #1		
Control	500 ± 206	
GM-CSF	4,246 ± 220	4,997 ± 491
CSF-1	NA	4,565 ± 868
GM-CSF (2x)	8,012 ± 638	10,234 ± 808
CSF-1 (2x)	NA	9,802 ± 925
Exp. #2		
Control	1,068 ± 28	
CSF-GM	ND	10,813 ± 344
CSF-GM (2x)	ND	19,507 ± 618
IL-3	12,753 ± 1,111	2,821 ± 969

NA = not available
ND = not done

TABLE III

FETAL ORIGIN OF THE PROLIFERATING CELLS IN THE PLACENTA

Group Number	Placental cell treatment	^{3}H TdR Uptake (cpm ± SD)	Percentage of Fetal Cells (± SD)
1	—	1,800 ± 190	67 ± 2.5
2	Rabbit Complement	427 ± 19	82 ± 1.5
3	anti H2-K^{k}	3,113 ± 65	63 ± 1
4	Rabbit Complement + anti H2-K^{k}	909 ± 44	88 ± 1
5	Rabbit Complement + anti H2-K^{k} + CSF-GM	11,024 ± 728	99 ± 0.8
6	CSF-GM	9,840 ± 240	73 ± 3

TABLE IV

Autoradiographic Determination of the Adherent Nature of Proliferating Cells

	% of Cells Incorporating ^{3}HTdR (± SD) Among:	
Treatment	Adherent Cells	Non Adherent Cells
--	0.4 ± 0.3	0
IL-3	3.7 ± 0.8	1.8 ± 0.4
CSF-GM (1X)*	7.2 ± 0.9	2.3 ± 0.5
CSF-GM (2X)#	33.7 ± 7.6	0.5 ± 0.3

* CSF-GM was added at beginning of culture

# CSF-GM was added at beginning and at 48 hours of culture.

TABLE V

Effect of rabbit-anti-mouse thymocyte serum and complement on the placental cell proliferation *in vitro*

	^{3}HTdR Uptake (cpm ± SD)	
Culture Conditions	Without CSF-GM	With CSF-GM
--	986 ± 138	11,970 ± 2,902
C'	568 ± 158	9,792 ± 954
ATS	1,897 ± 575	11,174 ± 1,583
C' + ATS	1,562 ± 592	9,531 ± 459
Spleen cells	Without PHA	With PHA
--	2,982 ± 949	28,690 ± 8,086
C'	257 ± 53	9,259 ± 561
ATS	2,516 ± 314	36,198 ± 4,430
C'+ ATS	2,591 ± 334	836 ± 255

TABLE VI

Effect of rabbit-anti-mouse thymocyte serum treatment on placental cell phagocytosis *in vivo*

	ATS treated mice	Control
OD at 414 nm ± SD	0.224 ± 0.039	0.449 ± 0.090
Number of Normal Placentae	72	56
Number of Mice	11	9
% of Phagocytic Cells	16 ± 8	37 ± 7

TABLE VII

IN VIVO EFFECT OF ANTI-LY2.1 ON PROLIFERATION AND PHAGOCYTOSIS OF THE INTACT PLACENTA

Treatment[1]	Proliferation/ Placenta (cpm ± SD)[2]	Phagocytosis/ Placenta (% ± SD)[3]
Control (No Treatment)	12,272 ± 1,945	37 ± 7
Control MoAb (28.8)[4]	11,409 ± 971	32 ± 4
Anti Thymocyte Serum	6,010 ± 1,114	16 ± 8
Anti Ly2.1 (HB 129)[5]	7,349 ± 1,049	12 ± 5

[1] C3H/HeJ females pregnant by Balb/cJ males were injected on days 8, 10 and 11 of pregnancy with the appropriate antibody.

[2] Proliferation is assessed by ^{3}HTdR uptake injected in vivo 24 h prior to sacrifice. Individual placentae were dissociated and counted in a β counter.

[3] Estimated as a percentage of fluorescent-bead positive cells among dissociated placental cells.

[4] 28.8 is an anti H-2k^b protein A purified IgG2a monoclonal antibody, not expected to react in this strain combination.

[5] Anti-Ly2.1 derived from ammonium sulfate precipitations of culture supernatants of the HB 129 hybridoma, which is specifically cytotoxic at the dilution of 1/100. This treatment did not significantly affect the rate of fetal resorptions (23% as compared to 11% of untreated controls).

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CHAPTER III

Fetally-derived Murine Placental Cells Simultaneously Display Trophoblast and Macrophage Characteristics [1]

INTRODUCTION

The most important maternal-fetal immune interactions take place in the placenta, a chimeric organ that contains fetal and maternal cells in direct contact with maternal circulation. This tissue is of obvious importance to the survival of the fetus, not only because it provides the nutrients and hormones necessary for embryonic development, but also because it serves as a partial barrier to cytotoxic maternal cells and antibodies directed against the fetus (1). The trophoblastic placenta originates from the outside layer of early embryonic cells and by day 11 has differentiated into an outer invasive spongiotrophoblast region, which is mixed with maternally-derived cells, and an inner labyrinthine zone, which is 100% fetal (2).

Recent experiments indicate that immunizing female mice with spleen cells related to the paternal type dramatically reduces the rate of fetal death (3). Such immunization leads to an increase in placental and fetal weight (4,5). We have therefore proposed that maternal T cells recognize fetal MHC class I antigens in the spongiotrophoblast and thereby cause increased placental growth and function through the release of lymphokines in the placenta (6). The generalization that T cells can cause non-lymphoid tissues to grow has been called immunotrophism (7). In experiments designed to test this hypothesis we showed that anti-thymocyte serum or monoclonal anti-T cell antibody depletion of maternal T cells during pregnancy results in decreased proliferation and phagocytosis in the intact placenta (8), as well as abortion in some strain combinations (Chaouat, G., E. Muno, I. Athanassakis and T.G. Wegmann, manuscript in preparation). In addition, we have isolated a lymphokine responsive fetal cell population from 12-14 day old murine placentas which is adherent, phagocytic, and non-specific esterase positive (FAPP cells). These cells proliferate *in vitro* in response to the T cell-derived lymphokines Interleukin 3 (IL-3), and granulocyte-macrophage colony stimulating factor (GM-CSF). They also respond to the monocyte-macrophage lineage-specific hemopoietic colony stimulating factor CSF-1 (8). The question arises as to whether FAPP cells are trophoblasts or mononuclear phagocytes.

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[1] This chapter has been submitted for publication. Irene Athanassakis, Simon Vassiliadis, Larry Guilbert and Thomas G. Wegmann. Journal of Immunology. 1988.

The objective of the current report is to determine whether FAPP cells represent a homogeneous or heterogeneous cell population and to determine whether they are macrophage or trophoblast in nature. Thus FAPP cells were reacted with a series of monoclonal antibodies that identify trophoblast- and monocyte-specific markers. In addition, differential dissection and gradient centrifugation were used to separately characterize the FAPP cells that reside in the spongiotrophoblast (invasive) and labyrinthine zones of the placenta. Here we report that FAPP cells consist of at least two populations: placental macrophages, and macrophage-trophoblasts which co-express what have been hitherto regarded as distinct macrophage and trophoblast cell surface markers.

MATERIALS AND METHODS

Timed Pregnant Mice

BALB/cJ and C3H/HeJ mice, 6 to 8 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME). Each C3H/HeJ or BALB/cJ female was examined vaginally for oestrus, caged overnight with a BALB/cJ male, and observed each morning for the presence of a vaginal plug. The day on which the plug was observed was designated day zero of pregnancy.

Antibodies

The rat IgG2b monoclonal antibody to murine/human Mac-1 antigen (M1/70HL, Hybritech, San Diego, CA), binds to macrophages at the optimal concentration of 0.5 μl/ml (1/200 dilution). The rat monoclonal antibody secreted by the F4/80 hybridoma is an IgG2a antibody directed against a 160K plasma membrane glycoprotein of mouse macrophages. It was used as a culture supernatant at a dilution of 1/10. The mouse IgG monoclonal antibodies to cytokeratin (PKK1, Labsystems, Helsinki, Finland) and vimentin (PK-V, Labsystems) were used at a dilution of 1/10. The anti-H-2D^d class I monoclonal antibody (IgG2a), produced by the 35-5-8S cell line (ATCC) and the anti-Ia^d class II monoclonal antibody (IgG2a) produced by the MKD6 cell line

(ATCC) were used at a dilution of 1/100. Protein A-purified mouse or rat IgG antibodies from normal serum, were also used as negative controls for the mouse- and rat-derived monoclonal antibodies at a concentration equivalent to that of the antibodies being tested.

Embryo Transfer

BALB/cJ females were induced to ovulate by injecting them i.p. with 5IU of pregnant mares serum (SIGMA, St Louis, Mo) followed 48 hours later by the i.p. injection of 5IU of human chorionic gonadotropin (SIGMA). Each female was paired with a BALB/cJ male 2 to 4 hours after the last injection. Blastocysts were obtained on the afternoon of the third day of pregnancy by flushing the isolated uteri with Leibowitz medium (Gibco, Grand Island, NY). Four to five blastocysts in Leibowitz medium supplemented with 5% fetal calf serum (FCS, Flow Laboratories, Mississauga, Ontario, Canada) were surgically transfered into each horn of anesthetized pseudopregnant C3H/HeJ females on day 2 of pseudopregnancy, timed from mating by vasectomized males.

Dissection of the Placenta

Pregnant mice were killed by cervical dislocation on days 12 to 14 of pregnancy. In some experiments the maternal circulation was flushed by injecting normal saline into the vascular system via an 18g needle inserted into the left aorta of anesthetized mice and held in place by forceps. Placentae were placed in Leibowitz medium, the remaining uterine tissue, visceral yolk sac and Reichert's membrane discarded and the decidual cap was carefully removed and stored at -70°C until GPI analysis was performed. The spongiotrophoblast, which is the white outer zone of the placenta, was separated from the labyrinthine trophoblast (red central zone) using fine iris scissors. Both preparations were frozen for GPI analysis.

In other experiments these tissues were made into single cell suspensions by repeated flushing through an 18G needle and fractionated using a discontinuous percoll gradient (see below). The cells recovered from each band of the gradient were either tested for responsiveness to growth factors or frozen for GPI analysis.

Fractionation of Placental Cells

Labyrinthine trophoblast, spongiotrophoblast or whole placental cell suspensions from either embryo-transfered or allogeneically-mated females were isolated on day 12 to 14 of pregnancy and fractionated on a percoll gradient. Isotonic percoll solution (Pharmacia, Dorval, PQ) was mixed with isotonic PBS to give final dilutions of 20, 40, 60 and 80% (1.02, 1.04, 1.06 and 1.08 g/ml). The gradient was prepared by layering the dilutions in a ten ml plastic centrifuge tube. Two milliliters of cell suspension containing a total of 50 x 10^6 cells were layered at the top of the gradient. After centrifugation at 600 xg for 30 minutes, the cells at the gradient interfaces were collected in Leibowitz medium and washed. Separate aliquots were tested for responsiveness and binding to growth factors, examined for surface markers with monoclonal antibodies, and analyzed (after freezing and thawing) for maternal versus fetal composition via the GPI isozyme assay described below.

Placental Cell Cultures

After removing the maternally-derived decidual layer of 12 to 14 day placentae, single cell suspensions were prepared by pushing the minced organ gently through a fine mesh wire screen into Leibowitz medium (Gibco). The cells were washed three times and cultured in RPMI 1640 (Gibco) supplemented with 10% FCS at 10^7 cells per ml in chamber slides (Lab-Tec, Miles Laboratories, Naperville, IL) or 35 mm plates (Falcon, Becton Dickinson, New Jersey) with various growth factors. In other experiments placental cells were fractionated on a percoll gradient and then cultured in 96 well plates (Linbro, Flow Laboratories, McLean, Virginia) with or without optimal doses of recombinant GM-CSF (Immunex, Seattle, WA, 5 pg/ml) or

CSF-1 (stage I, 9) added at the beginning and at 48 hours of culture. The cells were assessed for the incorporation of tritiated thymidine (^{3}HTdR) on day four of culture. One μCi of ^{3}HTdR (NEN, Ontario, Canada, specific activity 20 Ci/mmol) was added per well prior to harvest and the cells were placed in scintillation fluid (Toluene Omnifluor, NEN, Boston, MA, 1.38 g/l) and counted for beta emission in an LKB 1218 Rackbeta Counter. The results are expressed as an index of proliferation, which is: proliferation of treated cells (cpm)/proliferation of untreated control cells (cpm).

Phagocytosis was assessed by incubating the cells for 30 minutes with fluorescent beads (D=0.57 μ, Polysc. Inc. Warrington, PA), washing and reading under a fluorescent microscope. Cells were scored as phagocytic when more than 10 beads were seen in the cytoplasm.

GPI Analysis

Mice that are homozygous for the *Gpi-1b* allele express a dimeric isozyme that migrates faster in an electric field than the dimer expressed by *Gpi-1a* homozygous mice. Heterozygotes express both homodimers as well as a heterodimeric band with intermediate mobility. A mixture of homozygous and heterozygous cells does not allow easy quantitation by densitometry because of band overlap. To circumvent this problem, we transfered BALB/cJ

X BALB/cJ preimplantation blastocysts (*Gpi-1a*) into the uteri of pseudopregnant C3H/HeJ (*Gpi-1b*) females. The fetal and maternal GPI bands are thus electrophoretically unambiguous and lend themselves to sensitive quantitation using densitometry techniques. This assay has been described in detail elsewhere (10). Briefly, Gpi isozymes in the cell lysates were separated by using starch gel electrophoresis and then developed by a nitrocellulose overlay technique (10, 11). Quantitation of the relative contribution of maternal and fetal cells was obtained by scanning the stained nitrocellulose filters (mounted on microscope slides) using an Electrophoresis Scanner model 80100 (Camag, Muttenz, Switzerland). The signal produced was analysed by using a Hewlett-Packard model 3390A Integrator (Palo, Alto, CA), which determines the area of each band as a percentage of the total pattern. Artificial mixes of cells bearing the relevant isozymes generate a standard curve to which unknown samples can be compared. The sensitivity of the system is such that 1% of the minority isozyme can be detected in an artificial mixture. Each sample was run in triplicate.

Indirect Immunofluorescence

Placental cells were prepared as previously described and cultured in chamber-slides. The cells were stimulated at the beginning and at 48 hours of culture with an optimal concentration of partially purified GM-CSF (5ng/ml protein) kindly provided by Dr. C.R.Bleackley (Dept of Biochemistry, University of Alberta). After four days of culture the non-adherent cells were discarded and the adherent cells fixed in ice-cold methanol for 5 minutes and processed for immunofluorescence. After incubation in PBS supplemented with 1% normal goat serum at 4°C for 30 minutes, 0.2ml of the test antibody, diluted as described above, was added to each well and incubated for 45 minutes at 4°C. The cells were washed 5X with PBS. FITC-conjugated goat anti-mouse IgM, IgG or FITC-conjugated goat anti-rat IgG antibody (Cappel Laboratories, Cochranville, PA) was then added for 45 minutes at 4°C, followed by extensive washing with PBS. For double immunofluorescence experiments, the cells were prepared and stained with anti-Mac-1 or F4/80 followed by FITC-conjugated goat anti-rat antibody as described above. After washing with PBS the cells were further incubated with anti-cytokeratin for 45 minutes, washed and exposed to RITC-conjugated goat $F(ab')_2$ anti-mouse IgG antibody (Tago Inc. Burlingame, CA). Non-specific Fc binding was ruled out by using antibodies of the same isotype but of inappropriate specificity. In the negative controls no first antibody was added. There is only minimal cross reactivity between the goat anti-mouse antibody and the rabbit anti-rat immunoglobulin at the dilution used (see crosswise PBS controls, Table 2). Fluorescence was evaluated visually using a Zeiss fluorescent microscope.

^{125}I-CSF-1 Binding Assays

Methods for the preparation of purified CSF-1 and ^{125}I-CSF-1 have been described in detail elsewhere (9). In brief CSF-1 was labelled with ^{125}I using a modified chloramine T method to achieve a specific activity of 8x10^{17} cpm/mol of protein (9). Placental cells were cultured either in 35 mm Petri dishes (Lux Sc, Corp. Newburg Park, CA) or in chamber-slides as described above and stimulated at zero and 48 hours of culture with GM-CSF. On day 4 of culture the non-adherent cells were discarded and the adherent layer was cultured in growth factor-free medium for 24 hours to increase the CSF-1 receptor density and then washed twice with PBS in preparation for the binding reaction (12). Binding of ^{125}I-CSF-1 to adherent cells was carried out at 37°C, in an atmosphere of air containing 5% CO_2, in Iscove's medium (IMDM, Gibco) and at 4°C in IMDM minus bicarbonate titered to pH 7.35 with NaOH (ImB). Specific binding was determined as the amount of ^{125}I-CSF-1 binding that can be blocked by a two hour preincubation with 2nM unlabelled CSF-1 (9, 12). The binding reaction was stopped by 5 rapid washes of the adherent cells with ice-cold PBS. The cells in the plates were either solubilized with 5% SDS in 50 mM

Tris-HCl at pH 7.4 for counting in a LKB rack gamma counter, fixed in methanol at 4°C and processed for autoradiography, or maintained at 4°C for elution experiments.

^{125}I-CSF-1 Elution

Dissociation of cell-associated ^{125}I-CSF-1 (or its degradation products) from the adherent placental cells was carried out in 1ml of IMDM at 37°C or ImB at 4°C in 35 mm dishes. Medium was harvested (for ^{125}I counting) and replaced with fresh medium at 5, 15, 30, 60 and 90 min. At the end of 90 minutes the cells were solubilized and the remaining cell-associated radioactivity was counted.

Autoradiography

The slides were dipped in warm (42°C) emulsion (Kodak NTB2, Eastman Kodac Co., Rochester, NY), dried and enclosed in light-excluding containers for 5 and 10 days at 4°C. After warming at 22°C the slides were developed for 2 minutes in Kodak D-10 developer, rinsed for 30 seconds in 1% (V/V) acetic acid, fixed for 3 minutes in Kodak fixer and washed in five changes of distilled water. After drying, the preparations were stained with Giemsa at pH 6.5 and the percentage of cells with 10 or more silver grains above them was determined.

Bone Marrow-derived Macrophages

Single cell suspensions from femoral and tibial marrow cells were cultured in RPMI medium supplemented with 18% horse serum (Flow Laboratories) 2% FCS and 10% L-cell conditioned medium at 10^{6} cells/ml for 7 days. Macrophages were then harvested by 0.02% EDTA treatment at 37°C for 30min (13).

RESULTS

FAPP Cells Share Trophoblast and Macrophage Markers

We have previously isolated a lymphokine-stimulated FAPP cell population from the placentae of 12 to 14 day pregnant mice. By GPI analysis these cells are fetal in origin and display a number of characteristics typical of both macrophages or trophoblasts (8). They are adherent, phagocytic, stain positively for non-specific esterase, and they all respond to subclasses of the CSF family of growth factors (IL-3, GM-CSF and CSF-1) known to stimulate the growth of mononuclear phagocytes (14). CSF-1 has been described as specifically stimulatory of macrophage proliferation (15). In order to evaluate whether FAPP cells displayed macrophage or trophoblast characteristics we reacted them with antibodies directed against surface markers previously described as lineage specific for these two populations. Anti-Mac-1 is known to bind to the C3bi complement receptor (CR3) on granulocytes and macrophages (16). F4/80 binds to a membrane glycoprotein found only on mouse macrophages and its binding correlates with CSF-1 responsiveness (17). Cytokeratins are members of a multigene family present only on epithelial cells, whereas vimentin is the main subunit protein of intermediate filaments in fibroblastoid cells (18, 19). Trophoblast cells are known to stain positively with antibodies to cytokeratin but not vimentin (20, 21), whereas macrophages show the reverse pattern (19). The FAPP cells to be examined were obtained by stimulating C3H/HeJ x BALB/cJ placental cells after 12-14 days of gestation with an optimal dose of CSF-GM. The adherent layer was fixed in ice-cold methanol and processed for immunofluorescence. After background staining was subtracted, approximately half of the adherent cells stain positively for Mac-1 [3], F4/80 and cytokeratin antigens. They show only background levels of staining with antibodies specific for vimentin, as well as for class I and class II MHC antigens, whereas the latter antibodies stain the majority of bone marrow-derived macrophages (Table I). Non-specific binding was controlled for by using isotypically identical monoclonal antibodies that did not react with the FAPP cells. Rat IgG antibody used at a concentration equivalent to the protein content in the anti-Mac-1 and F4/80 preparation, as well as mouse IgG antibody used at the same concentration as anti-cytokeratin and anti-vimentin, showed only background levels of binding. Parallel experiments using BALB/cJ bone marrow-derived macrophages showed that these stain negatively for cytokeratin and positively for Mac-1, class I, class II MHC antigens and vimentin (Table I).

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[3] In a previous paper we reported in the discussion that FAPP cells were Mac-1 negative after harvesting with EDTA (8). However, we have subsequently found that harvesting the adherent cells with EDTA leads to a high background staining with the anti-immunoglobulin second antibody and that this background obscured the specific anti-Mac-1 interaction. Culturing the placental cells in chamber-slides allowed the adherent layer to be directly fixed and stained without need for harvest, thereby decreasing the background and revealing specific staining.

These results show that FAPP cells stain strongly for both macrophage and trophoblast markers and suggest that some of the cells may bear both markers. In order to determine whether both types of markers are indeed displayed on the same cells, we performed double immunofluorescence staining. The same cell population was reacted first with rat anti-Mac-1 or rat F4/80 antibodies and then with mouse anti-cytokeratin antibody using FITC anti-rat-Ig and RITC anti-mouse Ig-conjugated second antibodies for detection. All cytokeratin positive cells stained simultaneously with anti-Mac-1, and with F4/80 (Table II). The possibility that artifactual cross reaction between the FITC anti-rat Ig and the mouse anti-cytokeratin or between the RITC anti-mouse Ig and the rat F4/80 (or anti-Mac-1) can account for the double staining is excluded by controls in which PBS is substituted for either the F4/80 (or anti-Mac-1) or the anti-cytokeratin antibody in the double staining reaction (Table II).

Thus 50-60% of the lymphokine-stimulated adherent placental cells are (1) trophoblasts by the criterion of being positive for cytokeratin and negative for vimentin, and (2) macrophages by the criterion of Mac-1 and F4/80 marker expression. This is in contrast with bone marrow-derived macrophages, which in our hands are Mac-1, F4/80 and vimentin-positive but cytokeratin-negative (see Table I).

FAPP Cells Bind ^{125}I-CSF-1

FAPP cells proliferate in response to CSF-1, a growth factor restricted to the mononuclear phagocyte lineage at late stages of differentiation (8, 15). In order to determine whether this is a direct effect of CSF-1 on individual FAPP cells, we asked whether they bear the CSF-1 receptor, whether this receptor shares binding properties with the CSF-1 receptor on macrophages and whether the cells bearing the receptor grow in response to CSF-1. Placental cells were stimulated with GM-CSF or CSF-1 and after four days in culture the non-adherent layer was discarded and the adherent cells were cultured without growth factor for 24 hours to allow maximal expression of their CSF-1 receptors (12). ^{125}I-CSF-1 binding was examind at 4°C and 37°C, and at 15 minutes and 2 hours after adding the labelled CSF-1. The results show that specific binding occurs at both temperatures (Fig. 1A). After binding ^{125}I-CSF-1 the cells release very little radioactivity at 4°C whereas at 37°C appreciable ^{125}I is released (Fig 1B). These observations are in accord with published reports that at 37°C CSF-1 reversibly binds to specific cell surface receptors on macrophages and is internalized and degraded, whereas at 4°C the binding is essentially irreversible (9, 15). These results indicate that the FAPP population specifically binds CSF-1 and that the interaction is qualitatively the same as with macrophages.

In order to determine the fraction of the population able to bind CSF-1, CSF-1-stimulated FAPP cells were incubated with ^{125}I-CSF-1 for 2 hours at 4°C, washed, fixed and prepared for autoradiography. More than 60% of the FAPP cells were found to specifically bind CSF-1 (Table III). To determine whether the cells bearing CSF-1 receptors are the same as those proliferating in response to growth factors, ^{3}HTdR was added to the cultures 18 hours prior to CSF-1 binding. This allowed simultaneous examination of CSF-1 binding (silver grains over the membrane and cytoplasm of the cells) and proliferation (grains over the nucleus) (Fig 2). The results show that the same population that binds CSF-1 proliferates in response to CSF-1 (Table III). This conclusion also applies to GM-CSF-stimulated cells, which show similar capacity to bind ^{125}I-CSF-1 (data not shown). From these experiments we conclude that FAPP cells are directly stimulated by CSF-1 after it binds to the CSF-1 receptor on the cell surface.

Purification of FAPP Cells

These results indicate that the majority of FAPP cells have both macrophage and trophoblast characteristics. However, the low yield of cell numbers with the technique used above does not allow efficient characterization. It is especially important to determine whether FAPP cells are a uniform cell population or whether they are heterogeneous. We therefore fractionated freshly explanted placental cells with a percoll density step gradient. The cells collected from the 20-40% (40% band), 40-60% (60% band) and 60-80% (80% band) interfaces were tested for GPI phenotype, responsiveness to growth factors and marker expression.

The cells from the 40% band were large, vacuolated, sometimes multinuclear and represented approximately 65% of the total population. The cells isolated from the 60% band were smaller in size and their number corresponded to 5-10% of the total population. The cells isolated from the 80% band were small and represented about 25% of the total population (Table IV).

In order to determine whether the cells originate from the mother or the fetus, placental cells from embryo transfer-derived placentae (BALB/cJ x BALB/cJ blastocysts into C3H/HeJ pseudopregnant mothers, see methods) were fractionated on a percoll gradient and the recovered cells were frozen for GPI analysis. As shown in Table 4, the cells in the 80% band were 100% maternal in origin, whereas the cells of the 40% and 60% bands were 76 and 51% fetal in origin, respectively. Flushing out the maternal circulation by injecting normal saline into the left aorta of anesthetized mothers did not change the percentage of maternal cells in the placental cell preparation (data not shown). The percentage of fetal cells increased to 95 and 75% in the 40 and 60% bands, respectively, when the cells were cultured for 24 hours and only the plastic-adherent population was assayed for chimerism by GPI banding. Fluorescent bead uptake showed that cells in the 40 and 60% band were phagocytic, whereas the 80% cells were not (Table IV).

All cell populations proliferated (increased their level of incorporation of ^{3}HTdR) in response to GM-CSF when it was added at the beginning and at 48 hours of culture (Fig. 3). However, ^{3}HTdR uptake of the cells in the 60% band was 10 times higher than the cells in the 40% band (Fig 3).

To further characterize these two cell populations, we tested them for surface expression of Mac-1, F4/80, cytokeratin and vimentin as previously described. We observed that adherent cells derived from cultures of placental cells from the 40% band (FAPP40) express the same markers as FAPP cells derived from the unfractionated population: Mac-1, F4/80, cytokeratin-positive and vimentin-negative. This was seen regardless of whether they were stimulated with GM-CSF in culture (Table V). Adherent cells from the 60% band (FAPP60) stained with F4/80, and anti-Mac-1 in the absence of growth factor stimulation. However, they stained positively only with anti-Mac-1 antibody when cultured with GM-CSF. In both cases FAPP60 cells were negative for cytokeratin, which allows them to be classified as placental macrophages and distinguishes them from FAPP40 cells. The 80% band cells, 15% of which were adherent, were negative for all tested markers, since they stained to no greater extend than did the negative control.

Localization of FAPP Cells in the Placenta

The surface marker expression of FAPP40 cells suggest that these cells correspond to the initially described FAPP-macrophage-trophoblast cells. In order to determine whether FAPP40 cells are located in the spongiotrophoblast zone or in the labyrinthine trophoblast zone, the two layers were dissected from day 12-14 placentae and then fractionated with a percoll step gradient as described above. The fractionated cells were tested for GPI phenotype, responsiveness to growth factors and CSF-1 binding. GPI analysis was performed on tissue and on cell suspensions of the dissected placental tissues. Spongiotrophoblast consistenly yielded a smaller percentage of fetal cells in each band, as expected from the results of others (2). A pure fetal population was obtained from the 40% percoll band of labyrinthine trophoblast (Table VI).

All percoll fractions were further cultured in the presence of GM-CSF or CSF-1 and tested on day four for ^{3}HTdR uptake (Table VII). Cells in all fractions mitogenically responded to the growth factors by increasing ^{3}HTdR uptake over background levels. Relative to background (spontaneous) growth, cells in the 40% band showed a 3 to 12-fold increase of proliferation, and those in the 60% band showed a 2 to 4-fold increase. However, in terms of absolute ^{3}HTdR uptake, FAPP40 cells are much less responsive to the growth factors than FAPP60 cells (Table VII). The 80% cells also gave a proliferating response to CSF-1 and GM-CSF, but their maternal origin distinguishes them from the FAPP cells. In order to determine how many cells in each fraction bound CSF-1, cell surface binding of ^{125}I-CSF-1 at 4°C was assessed by autoradiography on adherent cells after 24 hours in culture. The results show that the majority of FAPP40 cells, and only a smaller proportion of FAPP60 cells bear the CSF-1 receptor (Table VIII).

Thus FAPP40 (macrophage-trophoblast) and FAPP60 (macrophage) cells are derived from both spongiotrophoblast and labyrinthine trophoblast. Cells from both fractions respond to GM-CSF and CSF-1 and contain cells bearing the CSF-1 receptor. However, FAPP60 cells grow much better than FAPP40 cells, even though they have fewer CSF-1-bearing cells. Interestingly, CSF-1 has the largest relative effect on the FAPP40 cell population because they do not proliferate in the absence of growth factor.

DISCUSSION

Our previous observations that adherent, phagocytic placental cells of fetal origin (FAPP cells) proliferated in response to lymphokines of the CSF familly suggested that either placental trophoblasts or fetal macrophages were the target for the immunostimulatory effects on placental growth observed in allogeneic pregnancies (8). FAPP cells were therefore examined for surface antigens characteristic of macrophages (Mac-1 or F4/80) and trophoblasts (cytokeratin). Although Mac-1 is considered a universal macrophage marker, it is also found on neutrophils (16). However, F4/80 appears to be specific to mononuclear phagocytes (17). Trophoblasts are typically characterized as a cytokeratin-positive and vimentin-negative fetal cell population (22). Double label immunofluorescence experiments with the macrophage markers and the trophoblast marker cytokeratin show that about half of the FAPP cell population bear both types of markers. Fractionation of placental cells on percoll step gradients prior to culture clearly separated these cells from the fetal macrophages, which do not stain for cytokeratin. These results show that FAPP cells are a heterogeneous population containing predominantly cells displaying both macrophage and trophoblast characteristics, some fetal macrophages, and very few cells bearing only the trophoblast marker. This conclusion is further supported by recent observations using FACS analysis, that long term cell lines derived from lymphokine-stimulated placental cells bear all the characteristics of the FAPP-macrophage-trophoblast cells described here (Mogil R., manuscript in preparation).

At least half of FAPP cells, therefore, are both macrophages and trophoblasts by the criteria of gross morphology, function (phagocytosis) and surface antigen expression. We have previously shown that FAPP cells proliferate in response to three factors known to stimulate macrophage growth: IL-3, GM-CSF and CSF-1 (8). IL-3 and GM-CSF are broadly acting lymphokines that stimulate the proliferation or differentiation of neutrophils, basophils, eosinophils, macrophages, erythrocytes, megakaryocytes and NK cells (23). However, the existance and binding kinetics of a receptor to CSF-1 appears to be restricted to mononuclear phagocytes (9, 14) and therefore serves as additional marker which is apparently specific for macrophages. We find that over half of FAPP cells bind ^{125}I-CSF-1 and respond to CSF-1 or GM-CSF and that the same receptor-bearing cells respond to these factors. Thus, even though fetal macrophages constitute only a small fraction of the total population, the majority of FAPP cells bear the receptor to the mononuclear phagocyte specific growth and differentiation factor CSF-1. These results support the above conclusion that the majority of FAPP cells bear markers specific to both macrophages and trophoblasts. They are also in accord with recent reports that the human choriocarcinoma cell line BeWo binds CSF-1 and expresses mRNA encoded by the proto-oncogene *c-fms*, the product of which is the CSF-1 receptor (24).

5

The immunofluorescence data show that 60 to 70% of FAPP cells stain positively for macrophage and trophoblast markers. Parallel experiments show that pure populations of bone marrow-derived macrophages stain 77% positive for Mac-1, whereas peritoneal macrophages stain only 45% positive for Mac-1 (data not shown). Thus, relative to the pure populations, the FAPP population stains as predominantly macrophage-trophoblast. However, immunofluorescence cannot determine whether minor subpopulations of cells bearing only single sets of markers are present. Placental cells were therefore fractionated on percoll step gradients in order to further purify the major population by separating out minor subpopulations of differing densities. The majority of placental cells appear in the 40% band and consist of Mac-1, F4/80, cytokeratin positive, and vimentin negative adherent cells (macrophage-trophoblasts). This procedure revealed a minor subpopulation of phagocytic, Mac-1, F4/80 positive, cytokeratin negative cells of fetal origin derived from placental cells banding at the 60% interface. By all the above criteria and responsiveness to CSF-1 and GM-CSF, these adherent cells are fetal macrophages. Indeed, the number of cells in the 60% band agrees well with the population of placental macrophages reported by other investigators (5-7%, 25, 26). The placental cells separating out at the 80% interface were neither of fetal origin nor phagocytic, and thus are not considered FAPP cells. Again very few adherent cells stain only with the trophoblast marker. Thus, the adherent placental cells reported by Zuckermann and Head (22) to display characteristics of trophoblast (cytokeratin positive, vimentin negative) may be macrophage-trophoblasts. However, these cells derive from cultures of denser placental cells (1.05 to 1.06 g/ml on percoll) than the FAPP40 cells (1.02 to 1.04 g/ml) and are negative for phagocytosis (as measured by zymosan particle uptake). They are also not stimulated by lymphokines or cytokines, in contrast to the cells studied here.

The observation that all the macrophage-trophoblasts, but none of the fetal macrophages or adherent maternal cells, derive from placental cells banding at 40% percoll allowed us to determine whether macrophage-trophoblasts

(FAPP40 cells) were isolated from the labyrinthine or spongiotrophoblast zone. Dissection of the two zones prior to percoll separation and adherence selection shows FAPP40 cells are present in both zones but the majority is isolated from the labyrinthine trophoblast. Approximately equal numbers of fetal macrophages (FAPP60 cells) come from the two zones; however, no adherent cells of maternal origin (FAPP80 cells) derive from the labyrinthine trophoblast. FAPP40 cells originating in both zones respond in similar fashion to the same growth factors. Thus, growth factor responsiveness does not distinguish the macrophage-trophoblasts found in the two trophoblast zones. However, our studies have been concentrated on relatively mature placental cells. Fenderson et al. have described a system where peritoneal exudate cells from multiparous but not virgin females stimulate blastocysts to incorporate ^{125}IUdR (27). In contrast, Hill et al. showed that activated-lymphocyte or macrophage products could inhibit blastocyst formation from two-cell stage embryos (28). Therefore, the growth factor dependency of trophoblasts depends on the developmental stage of these cells.

Interestingly, once separated from the other two subpopulations, FAPP40 cells adhere in culture and bear the CSF-1 receptor but grow very slowly.

Macrophage-trophoblasts (FAPP40 cells) from the labyrinthine trophoblast account for almost 65% of the total population and over 80% of CSF-1 receptor-bearing cells in the placental cultures, yet account for less than 4% of the proliferative response to CSF-1 (calculated from Tables VII and VIII). Most of the response to CSF-1 in separated cultures comes from fetal macrophages (FAPP60 cells) and adherent cells of maternal origin banding at the 80% percoll interface which together account for less than 2% of the CSF-1 receptor-bearing population. In unseparated cultures that are lymphokine-stimulated, macrophage-trophoblasts constitute about 60% of the FAPP cells and all appear to bear the CSF-1 receptor and respond to CSF-1. Thus, it appears that macrophage-trophoblasts require not only macrophage growth factors to proliferate but also something provided by other placental cells present in the unseparated cultures. For example, fetal macrophages may respond to CSF-1 by elaborating placental growth factors such as the B chain of PDGF (29), which in turn has been shown to be a trophoblast growth factor (30). Placental trophoblast stimulating factors have also been shown to be produced by maternal T lymphocytes and in particular by decidual cap cells (manuscript in preparation). These observations argue for the existance of a growth regulatory network involving several placental cell types of both maternal and fetal origin.

Finally, these results indicate that whatever the net effect of the maternal immune response is on fetal survival and growth, there exists an important stimulatory component of the response which can be quantitated and which is mediated by a novel phagocytic cell of fetal origin that shares the properties of macrophage and trophoblast. Thus the results are in agreement with the immunotrophism hypothesis, according to which paternal alloantigens stimulate maternal T cells to secrete products that create an environment favorable to placental growth, which in turn can lead to improved fetal survival.

Acknowlegments

We thank D. Branch for help with the manuscript and M. Ritzel for providing us the bone marrow-derived macrophages.

Figure 1

Specific binding of ^{125}I-CSF-1 to FAPP cells.

A. Adherent placental cells were incubated with ^{125}I-CSF-1 at 4°C or 37°C for 15 min and 2 hours and specific binding determined as described in the methods. Bars denote standard deviation.

B. Dissociation of cell bound ^{125}I from FAPP cells after 2 hours of incubation with ^{125}I-CSF-1 at 4°C (■) or 37°C (●). Dissociation was carried out at the same temperature as the incubation ans is expressed as the percent of specific ^{125}I-Release.

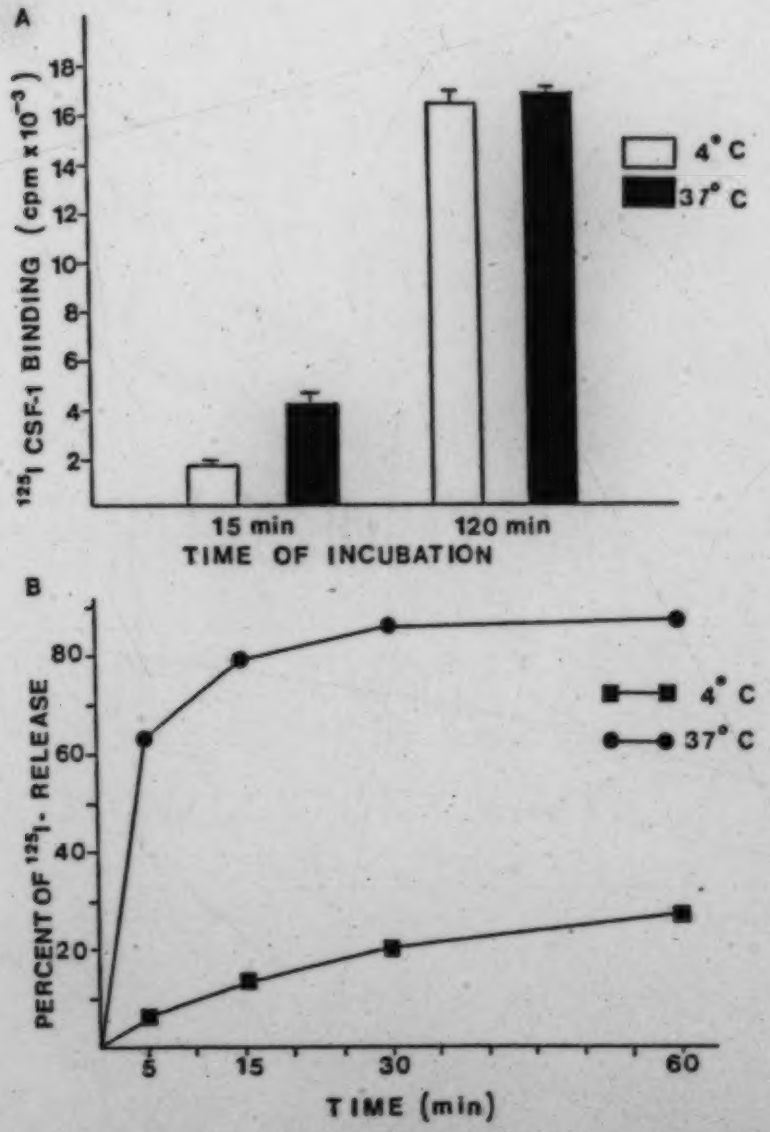

Figure 2

Double autoradiography showing CSF-1 binding and ^{3}HTdR uptake on FAPP cells. A. ^{125}I-CSF-1 binding. B. ^{3}HTdR uptake showing one cell in the S phase and one in anaphase. C. ^{125}I-CSF-1 binding and ^{3}HTdR uptake seen on the same cell. The dark nucleus corresponds to ^{3}HTdR incorporation and the dotted membrane and cytoplasm to CSF-1 binding.

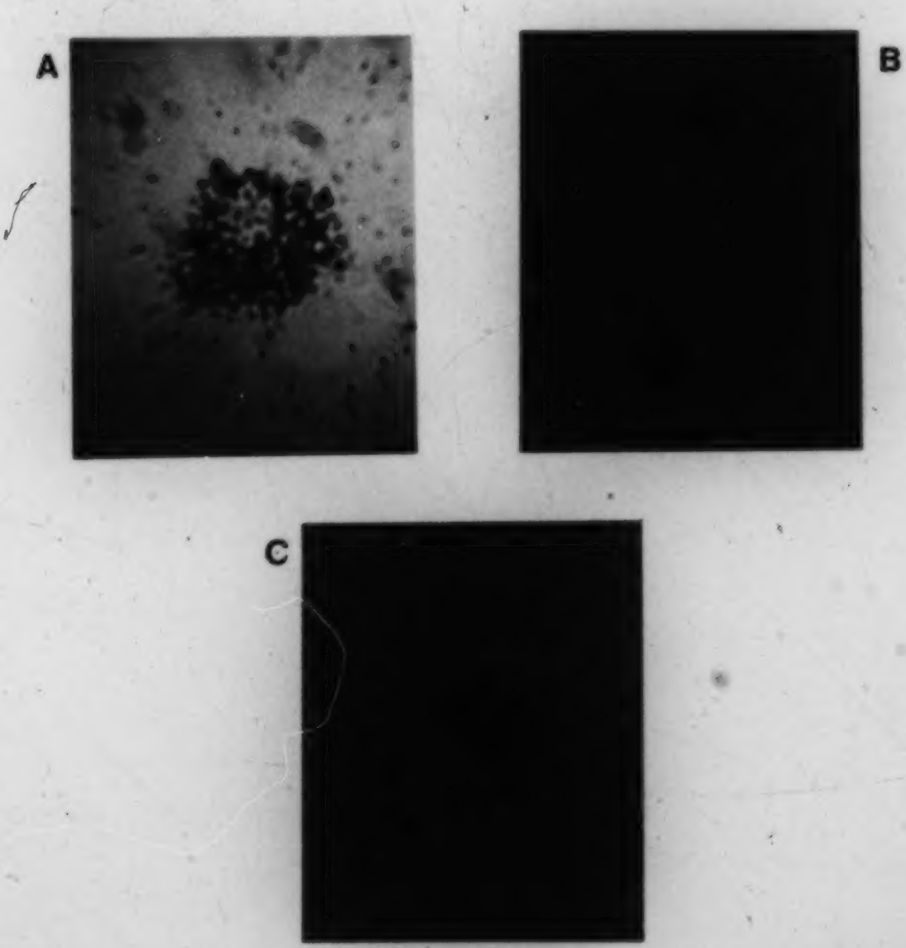

Figure 3

Index of ^{3}HTdR uptake in response to GM-CSF on Percoll fractionated placenta. The placental cells were fractionated using a density step gradient consisting of the following percentages of percoll: 40% (20-40% interface), 60% (40-60% interface) and 80% (60-80% interface). The cells were stimulated with an optimal dose of GM-CSF at the beginning and at 48 hours of culture and ^{3}HTdR uptake was assessed on day 4 of culture. The results are expressed as an index of proliferation = treated cells/control, where the control corresponds to the unstimulated groups. The respective values of controls in net cpm/10^6 cells are: 40%: 831 ± 107, 60%: 870 ± 359, 80%: 92 ± 6.

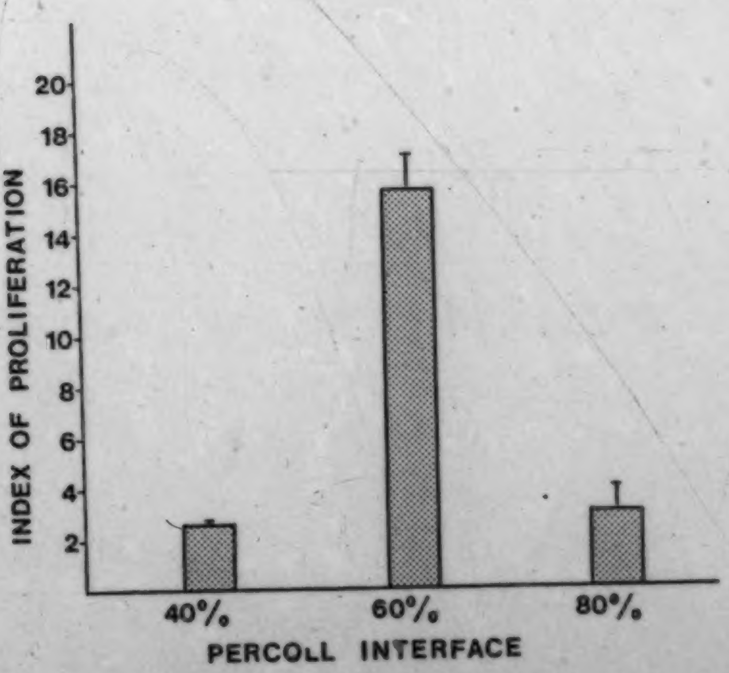

TABLE I

Macrophage and Trophoblast marker expression on FAPP cells tested by immunofluorescence.

Antibodies directed against	% positive cells FAPP cells
Mac-1	79±4
F4/80	69±2
Cytokeratin	74±9
Vimentin	10±2
Class I D^d	32±7
Class II Ia^d	19±8
Mouse IgG*	11±4
Rat IgG+	8±1
--	5±1
	Bone marrow-derived macrophages
Mac-1	86±8
F4/80	93±7
Cytokeratin	12±5
Vimentin	84±9
Class I k^d	83±7
Class II Ia^d	79±2
Mouse IgG*	4±2
Rat IgG+	9±1
--	8±2

* Mouse IgG antibodies were used as negative controls for mouse anti-cytokeratin, anti-vimentin, anti-class I and anti-class II antibodies.

+ Rat IgG was used as negative control for anti-Mac-1 and F4/80, which are rat antibodies.

TABLE II

Double staining on fixed FAPP cells.

	Percent of cells that stain for		
Antibody combination	FITC[1]	RITC[2]	FITC+RITC
Mac-1	78±9	--	--
Cytokeratin	--	77±7	--
F4/80	75±4	--	--
PBS[3]	12±6	16±3	--
Mac-1^F + Cyt^R	60±5	68±6	60
Mac-1^F + PBS^R	72±5	6±1	4
F4/80^F + Cyt^R	73±3	65±2	65
F4/80^F + PBS^R	70±4	7±1	5
PBS^F + Cyt^R	10±3	71±4	6
PBS^F + PBS^R	17±2	14±1	5

1: Rabbit anti-rat FITC conjugated antibody was used to detect the rat anti-Mac-1 and F4/80 monoclonal antibodies.
2: Goat anti-mouse RITC conjugated antibody was used to detect the mouse cytokeratin monoclonal antibody.
3: Control group where antibody was replaced by phosphate buffer.
F: FITC; R:RITC.

Double stained cells were first stained with anti-Mac-1 and/or F4/80 using FITC, then stained with cytokeratin detected by RITC:

F4/80 (F)

Cyt (R)

TABLE III

Autoradiographic analysis of CSF-1 binding by CSF-1 stimulated cells.

Treatment	% cells with grains above:	
	nucleus	cytoplasm/membrane
^{125}I-CSF-1[a]	--	72 ± 5
^{125}I-CSF-1 + CSF-1[b]	--	9 ± 1
^{3}HTdR	67 ± 1	--
^{3}HTdR + ^{125}I-CSF-1[c]	67 ± 10	62 ± 14

a) The nucleus or cytoplasm/plasma membrane of a cell was scored positive when more than 10 grains were seen above it.
b) Preincubation blocking by 2nM unlabelled CSF-1.
c) The CSF-1 stimulated adherent cells were first incubated with ^{3}HTdR and then processed for ^{125}I-CSF-1 binding.
The cells having silver grains over both the nucleus and cytoplasm were considered double positive.

TABLE IV

GPI analysis and phagocytosis of placental cells fractionated on Percoll.

Interface	% total cells	Fetal cells A + NA (%)	Fetal A (%)	Phagocytosis A (%)
40 %	67± 9	76±2	95±2	100
60 %	6± 3	51±5	73±7	100
80 %	26±12	0	0	0

A: adherent cells
NA: non-adherent cells

TABLE V

Marker expression on the adherent cells[1] derived from percoll-fractionated placental cells.

Percoll interface	GM-CSF[2]	Mac-1	F4/80	Cytok.	Vim.	control (no 1st Ab)
40%	+	78±7	87±4	83±5	27±4	27±4
60%	+	67±1	29±4	33±8	35±7	26±8
80%	+	18±6	20±4	18±5	12±5	21±5
40%	-	79±13	92±1	92±8	33±1	24±4
60%	-	68±8	65±4	25±8	15±4	16±4
80%	-	13±3	17±6	14±2	9±1	22±4

1: Marker expression was tested on the adherent cells on day 4 of culture by immunofluorescence.

2: Cells exposed to 5ng/ml of GM-CSF at the begining and 48 hours of culture.

TABLE VI

GPI analysis on dissected placenta.

	% FETAL CELLS	
	SPONGIO	LABYRINTHINE
Tissue	86±6	100
Cell Susp.	63±8	74±8
40% Percoll	89±3	99±1
60%	30±2	73±5
80%	0	--[1]

	% OF TOTAL CELLS	
40% Percoll	15±5	64±6
60%	3±1	4±1
80%	11±5	--[1]

1: No cells appear in the 80% band from the labyrinthine.

The 40, 60 and 80% correspond to the Percoll 20-40%, 40-60% and 60-80% interfaces.

TABLE VII

Proliferation of fractionated spongiotrophoblast and labyrinthine trophoblast in response to GM-CSF and CSF-1.

	Net stimulation[a] per 10^6 cells (cpm)[b]	
	CSF-1	GM-CSF
Lab[c] 40%	7,336±1,547	1,570± 27
Lab 60%	69,500±8,200	36,300±3,133
Sp[d] 40%	2,500± 965	1,338± 688
Sp 60%	49,767±4,367	18,500±3,367
Sp 80%	73,000±4,600	31,564±3,700

a. Cells were stimulated with optimal doses of GM-CSF and CSF-1 at the begining and at 48 hours of culture, and proliferation was assessed on day 4. 1uCi of ^{3}HTdR was added per well 18 hours prior to harvest.

b. The respective values of untreated controls in cpm/10^6 cells are: Sp 40%, 762±87; Sp 60%, 17,100±6,833; Sp 80%, 15,900±5,700; Lab 40%, 657±80; Lab 60%, 34,033±13,133.

c. Lab: labyrinthine trophoblaswt

d. Sp: spongiotrophoblast.

TABLE VIII

Autoradiography of CSF-1 binding.

	Percent of labelled cells	
	HOT	HOT + COLD[3]
Lab[1] 40%	97±3	29±3
Lab 60%	30±4	16±4
Sp[2] 40%	70±6	19±3
Sp 60%	47±7	31±5
Sp 80%	16±3	4±1

1: Lab=labyrinthine.
2: Sp=spongiotrophoblast.
3: Binding not blocked by cold CSF-1 used at saturating conditions.

40, 60 and 80% correspond to the 20-40%, 40-60% and 60-80% Percoll gradient interfaces.
Cells were incubated with ^{125}I-CSF-1 for 2 hours at 4°C, washed, fixed and processed for autoradiography.

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FOOTNOTES

Abbreviations used

GM-CSF: granulocyte-macrophage colony stimulating factor; IL-3: interleukin 3; CSF-1: colony stimulating factor-1; FAPP: fetal adherent phagocytic placental cells.

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Grant and Financial Support

This work was supported by the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

CHAPTER IV

The Effect of Maternal T Cells on Placental Growth and Decidual Lymphokine Production in Allogeneic and Syngeneic Murine Pregnancy [1]

INTRODUCTION

The mammalian placenta is a unique chimeric organ in which fetal trophoblast cells, bearing class I MHC antigens, come into direct contact with maternal decidual cells as well as maternal circulation. Recently, it has become clear that there is maternal immune recognition of fetally-derived allogeneic tissue in the placenta during normal pregnancy (1). Thus allogeneic pregnancies show a greater influx of maternal T cells into the decidua than do syngeneic pregnancies (2,3,4), with the peak response occuring shortly after the appearance in ontogeny of Class I expression in the placenta (3). This is also reflected in increased draining lymph node size and macrophage inhibition factor release in allogeneic versus syngeneic pregnancies (5,6,7,8). Such maternal immune recognition has consequences for fetal survival, because there are cases of spontaneous abortion in which immunization of the mother with white cells related to paternal type can dramatically improve the probability of fetal survival (1,9,10). One explanation of how maternal immunological activity may improve placental function and fetal survival is called immunotrophism. It postulates that maternal T cell recognition of the semi-allogeneic placenta leads to lymphokine and cytokine release at the maternal-fetal interface which in turn stimulates trophoblastic growth and improves the chances of fetal survival by somehow improving placental function (11).

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[1] This chapter has been submitted for publication. Irene Athanassakis, Donald R. Branch, Maria I. Garcia Lloret, Larry Guilbert and Thomas G. Wegmann. Journal of Immunology. 1988.

In our previous work we have subjected this hypothesis to two different sets of experimental verification, one *in vitro* and the other *in vivo*. We showed, first of all, that fetally-derived placental cells grow in tissue culture in response to T cell-derived lymphokines of the CSF family, including GM-CSF and IL-3 (12). They also respond to the cytokine CSF-1 (12), the levels of which are increased in the uterus by the hormonal changes associated with early pregnancy (13). We have subsequently identified the cells that respond to these molecules as trophoblasts because they are cytokeratin-positive and vimentin-negative (14, Athanassakis submitted for publication[1]). We have also achieved long term trophoblast growth using lymphokine supernatants (Mogil et al. submitted for publication[3]). A second line of experimental test has involved removing maternal T cells in mid-gestation to examine what effect this has on placental growth and function. We have already reported that treatment of pregnant female mice during mid-gestation with anti-thymocyte serum as well as anti-CD8 (Ly2.1) monoclonal antibody leads to a two-fold reduction in placental proliferation and phagocytosis *in vivo* (12).

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[1] Athanassakis et al. 1988. Fetally-derived murine placental cells simultaneously display trophoblast and

In the current report we have extended this set of observations by showing that maternal T cell depletion *in vivo* by monoclonal antibodies directed against T cell determinants not only affects allogeneic pregnancies, but can also reduce the proliferative and phagocytic capacity of syngeneically pregnant mice as well. In both types of pregnancy we find that anti-CD8 antibody injection leads to a reduction of placental proliferation and phagocytosis. Anti-CD4 antibody, on the other hand, only affects placental proliferation, indicating that there is a complex network of immune interactions affecting placental growth and function. In an attempt to localize

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[2](cont'd) macrophage characteristics, submitted for publication.

[3] Mogil 1988. Immunoregulatory effects of lymphokine-driven placental cells on cloned T cells, submitted for publication.

the source of this T cell-mediated effect, we have established decidual cell cultures and have found that supernatants from these cultures have the capacity to stimulate trophoblast cell growth. To determine the nature of the substances within these culture supernatants we added them to cell lines which are differentially sensitive to members of the CSF family of lymphokines. We report here that these decidual supernatants can stimulate proliferation in the cell line DA-1, which is responsive to both IL-3 and GM-CSF (15). Allogeneic supernatants are more potent in this regard than syngeneic supernatants, and both show ontogenetic patterns which correlate with the pattern of maternal T lymphocyte influx into the decidua. Mid-gestational T cell-depletion by monoclonal antibody injection lowers the capacity of both allogeneic and syngeneic decidual cells to produce these growth factors in tissue culture. Finally, we report that treating the supernatants with antibody specific to GM-CSF completely eliminates such growth activity, indicating that GM-CSF is released from decidual supernatants, and that allogeneic decidua has a greater capacity to produce GM-CSF than does its syngeneic counterpart. Both types of decidual supernatant also contain CSF-1 activity, a result that confirms and extends the work of Stanley and his collegues (13). The current results provide further evidence in support of the placental immunotrophism hypothesis as applied to allogeneically mated mice, and extend it to syngeneic pregnancy as well.

MATERIALS AND METHODS

Mice

BALB/cJ (H-2K^d) C3H/HeJ (H-2K^k) 6-8 weeks old were obtained from Jackson Laboratories (Bar Harbor, ME). Each C3H/HeJ female was checked for oestrus, caged overnight with a BALB/cJ or C3H/HeJ male and checked for the presence of a vaginal plug on the following morning. The day on which the plug was observed was considered to be day zero of pregnancy.

Cell Cultures

Placentae were obtained from females on day 12 to 14 of pregnancy. After removing the maternally-derived decidual layer, single cell suspensions were prepared by pushing the placentae through a fine mesh wire screen into Leibowitz medium (Gibco, Grand Island, NY). The cells were washed three times and cultured in RPMI 1640 (Gibco) supplemented with 10% FCS at 10^7 cells per ml in 96 well plates (Linbro, Flow Labs, Mc Lean, Virginia) with or without various culture supernatants. Cultures were assessed for ^{3}HTdR incorporation two days later. One μCi of ^{3}HTdR was added per well prior to harvest and the cells were placed in scintillation fluid (toluene omnifluor, NEN, Boston, MA, 1.38 g/l) and counted for beta emission in an LKB 1218 Rackbeta Counter.

Decidual layers were isolated from days 11, 12, 13 and 14 of pregnancy, put into cell suspension by pushing the tissue through a mesh wire screen, washed and cultured in RPMI 10% FCS at $5x10^6$ cells/ml in 24 well plates (1ml/well). Culture supernatants were collected from day one to day six of culture, and tested for their ability to induce proliferation of placental cells and DA.1 cells. Spleen culture supernatants from day 12 of pregnancy were prepared the same way as described for the decidual cap cells.

Cell lines

Murine DA-1 cells are dependent upon Interleukin-3 (IL-3) for growth and also respond to GM-CSF and erythropoietin (EPO) (15,16,17). DA-1 cells do not proliferate in response to CSF-1 (15). The cells were routinely cultured in suspension in Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS, Flow Laboratories, Mississauga, ONT) plus 20% medium conditioned by WEHI-3 cells (American Type Tissue Culture collection, Rockville, MD). Bioassays using DA-1 cells were performed using microtiter plates as previously described (15) with slight modification. 25 µl of each culture supernatant was added to the 96-well flat-bottom microtiter plates (Linbro, Flow Laboratories) to give a final concentration of 50% v/v. Each well contained 10^4 DA-1 cells suspended in 5% (routine) or 15% (anti-GM-CSF blocking studies) IMDM to give a final volume of 50 µl. The plates were incubated for 24 (anti-GM-CSF blocking studies) to 48 hours (routine). Proliferation was assessed by adding 1µCi ^{3}H-Thymidine (^{3}HTdR NEN, Boston MA) per well 4 hours prior to harvest, and counting radioactive uptake using gamma scintillation. Positive controls consisted of WEHI-3CM (50%), pure natural murine IL-3 (300 U/ml, Genzyme, Boston, MA), pure recombinant murine GM-CSF (10 pg/ml or 50 ng/ml, a gift from Dr. R.J. Tushinski, Immunex, Corp. Seattle, WA) and pure recombinant human erythropoietin (5 U/ml, Lot P004, Genetics Institute, Boston, MA). Negative controls consisted of medium alone. Rabbit anti-mouse GM-CSF (anti-GM-CSF) used in inhibition experiments was a gift from Dr. J.F. Delamarkter, Biogen, Geneva, Switzerland. Anti-GM-CSF or normal rabbit serum was mixed with positive or negative control culture supernatants to give a final concentration of 2% v/v. The mixture was incubated for 2 hours at 37°C in the microtiter wells prior to addition of DA-1 cells. Assays were then incubated for an additional 20 hours prior to pulsing with ^{3}HTdR and harvesting.

T Cell Depletion in vivo

C3H/HeJ females pregnant by BALB/cJ or C3H/HeJ males were injected on days 8, 10 and 11 of pregnancy with 0.5 mg of anti-L3T4 and/or anti-Ly2.1 intraperitoneally (i.p.). Anti-L3T4 (GK1.5, ATCC Rockville, Maryland) and anti-Ly2.1 (H129, ATCC) were used as concentrated supernatants for *in vivo* injections. This treatment specifically eliminated L3T4 or Ly2.1 positive cells from maternal spleen as tested by cytotoxicity and immunofluorescence (Table I). For the cytotoxicity assay 10^6 spleen cells from treated mice were incubated with anti-L3T4 or anti-Ly2.1 antibodies for 30 minutes at 37°C, then washed and incubated for 30 minutes at 37°C with Low Tox rabbit complement (1/10 v/v, Cedarlane, Hornby, ONT.). Killing was evaluated by light microscopy after trypan blue staining. For the immunofluorescence assay, spleen cells were incubated with anti-L3T4 or Ly2.1 antibodies at 4°C for 30 minutes, washed and exposed to FITC-conjugated goat anti-rat Ig antibody (Cappel, West Chester, PA) for 30 minutes at 4°C. The percentage of fluorescent cells was evaluated using a Zeiss fluorescent microscope.

Placental proliferation was assessed *in vivo* by injecting the mice on day 12 of pregnancy with 27μCi of ^{3}HTdR i.p.. The following day the mice were killed by cervical dislocation and placentae and spleens were removed. Individual placentae were cut into small pieces using fine scissors, placed in 3ml of NCS-ACS solution (1:10, Tissue solubilizer/aqueous counting scintillant, Amersham, Oakville, ONT), shaken thoroughly and counted in an LKB beta counter. Each spleen was individually made into a cell suspension and tested for ellmination of L3T4 or Ly2.1 positive cells by complement-mediated cytotoxicity and by immunofluorescence as described above.

Phagocytosis was assessed on cells derived from the 12 day placentae. The suspended cells were incubated with fluorescent beads (D=0.57 μ, Polysciences Inc. Warrington, PA) for 30 minutes at 37°C and washed. Phagocytosis was estimated visually with a fluorescent microscope. A cell was considered phagocytic when more than 20 beads were seen in the cytoplasm.

RESULTS

T Cell Depletion in vivo Affects Placental Proliferation and Phagocytosis in both Allogeneic and Syngeneic Pregnancies

We have previously shown that maternal T cell depletion by rabbit anti-mouse thymocyte serum (ATS) or anti-CD8 (Ly2.1) monoclonal antibody treatment during allogeneic pregnancy results in a two-fold reduction in placental proliferation and phagocytosis *in vivo* (12). CD8-positive T cells represent only one subset of the T cell population, the other major subset being CD4-positive T cells. We have therefore treated pregnant mice with both anti-CD4 (anti-L3T4) and anti-CD8 (anti-Ly2.1) monoclonal antibodies on days 8, 10 and 11 of pregnancy. An interesting difference emerges between treatment with the two different types of antibodies, as shown in Table II. Both anti-CD4 and anti-CD8 treatment have an equal effect in reducing placental proliferation, and the combination of the two antibodies is no more potent in this effect than the two separate treatments. A clear cut difference emerges when one studies the phagocytic capabilities of the placenta *in vitro*, following *in vivo* treatment. As we have reported previously, the anti-CD8 antibody causes a clear reduction in the phagocytic capability of the placenta. However, phagocytosis is not affected by anti-CD4 antibody.Neither treatment affected fetal viability or placental weight in this strain combination.

Allogeneically pregnant mice have larger placentae and uterine draining nodes than do syngeneically pregnant mice, as well as a greater influx of T cells into the decidua following MHC Class I gene expression on the invasive trophoblast. Nevertheless, syngeneically pregnant mice do show some evidence of maternal T cell influx into the decidua (18). This could be due to the expression of unusual Class I MHC antigens or minor histocompatibility antigens to which the mother is not tolerant (23). These observations led us to inquire whether deletion of maternal T cells in syngeneically pregnant mice would affect placental proliferation and phagocytosis as it does in allogeneically pregnant mice. To address this question we injected C3H female mice pregnant by C3H male mice with the anti-CD4 and CD8 monoclonal antibodies described above, using the same timing and dosage. The results, shown in Table III, indicate that control syngeneically pregnant mice (C3H x C3H) have a lower tritiated thymidine uptake per placenta than allogenically pregnant mice, but it is approximately equal to that seen in allogeneic pregnancy when the uptake is expressed as a function of placental weight. Thus, the apparent difference simply reflects a difference in placental size in the two situations. Treatment with anti-CD4 and anti-CD8 antibodies had virtually identical effects to those seen in allogeneically pregnant mice. Both antibodies reduced placental proliferation, while only the anti-CD8 antibody showed a significant effect on placental phagocytosis. Once again, there was no obvious effect on either placental weight or fetal survival. These results lead to the conclusion that maternal T cells play a role in placental proliferation whether or not the fetus is allogenic to the mother. CD8 T cells in both instances seem to be important for stimulating placental proliferation and phagocytosis, whereas CD4 T cells appear to play a comparable role in stimulating placental proliferation but not in promoting placental phagocytosis. These results focus attention on local immunoregulatory events in the decidua and led us to examine the effects of maternally-derived decidual cell supernatants on the growth of placental trophoblast and lymphokine-sensitive cells.

Decidual Supernatants Contain Trophoblast Growth Factors

We have previously shown that lymphokines and cytokines of the CSF family have an effect on trophoblastic proliferation *in vitro* and the results presented above indicate that maternal T cell deletion during pregnancy has an effect on placental proliferation in both syngeneic and allogeneic pregnancies. In order to localize this effect, we next determined whether decidual cells derived from such pregnancies will release substances that can influence placental cell growth. Thus we made decidual cell suspensions at day 12 of pregnancy from both C3H x BALB and C3H x C3H matings. The cell suspensions were placed in tissue culture and the supernatants were collected from days one to six of culture. These were individually assessed for their ability to stimulate proliferation in placental cell cultures, by tritiated thymidine uptake. The results in Figure 1 show that both allogeneic and syngeneic supernatants cause placental cell proliferation. The effect is specific for supernatants derived from the decidual cell cultures, because spleen cell suspensions made from the same pregnant females have no effect on placental cell proliferation (see legend, Figure 1). Stimulation of placental cells by GM-CSF, which we have previously shown to be a trophoblast cell growth factor, is included as a positive control. Having found that the activity of decidual cell supernatants can cause placental cells to proliferate, the next question we addressed was the nature of the growth factors having this effect.

Growth Factors Present in Decidua Supernatants Affect the Lymphokine-Sensitive Cell Line DA-1

DA-1 cells are known to proliferate in response to IL-3, GM-CSF, and erythropoietin, but not to CSF-1 or IL-4 (15,17). We therefore cultured decidual cells taken from days 11, 12, 13 and 14 of allogeneic (C3H x BALB) or syngeneic (C3H x C3H) pregnancy. Cultured supernatants were collected daily and were individually tested for their capacity to stimulate proliferation of the DA-1 cells. As can be seen in Figure 2, both allogeneic and syngeneic decidual cell supernatants stimulate the growth of the DA-1 cells, but the allogeneic supernatants had a more pronounced effect, particularly on days 11 and 12 of pregnancy. This pattern closely correlates with the histological appearance of T lymphocyte influx into the decidua as described by others, both in rank order of allogeneic versus syngeneic pregnancy, and with respect to maximal response on a given day of pregnancy. This correlation between T cell migration into the decidua and growth factor release from decidual cells in culture raises the question of whether the growth factor production is dependent upon the integrity of the maternal T cell system.

Maternal T Cell-depletion in vivo Reduces Growth Factor Production by Decidual Cells

In order to determine whether decidual growth factor production is a function of maternal T cells (19,20), we treated allogeneically (C3H x BALB) and syngeneically (C3H x C3H) pregnant mice with anti-CD4 and anti-CD8 monoclonal antibodies on days 8, 10 and 11 of pregnancy, as described above. We examined day 12 decidual cells to see whether they could release growth factors capable of stimulating the proliferation of DA-1 cells. The results, shown in Figure 3, indicate that such treatment significantly reduces the capacity of both allogeneic and syngeneic decidual cell supernatants to promote ^{3}HTdR uptake by DA-1 cells. These results indicate that maternal T cells in both allogeneically and syngeneically pregnant mice are necessary for growth factor production by decidual cells. In addition this observation strengthens the correlation between histological appearance and growth factor production cited above. These results led us next to inquire whether T cell-derived lymphokines might be the growth factors present in the decidual cell supernatants.

The Stimulatory Activity of Decidual Supernatants on DA-1 Cells Is Primarily Due to GM-CSF

As stated previously, the DA-1 cell line is sensitive to IL-3, GM-CSF and erythropoietin, but not to CSF-1. We therefore inquired whether treatment of pooled decidual cell supernatants with rabbit anti-GM-CSF polyclonal antibody could block DA-1 cell proliferation. As shown in Figure 4, the presence of anti-GM-CSF antibody blocked the stimulatory activity of both allogenic and syngeneic supernatants of 13-day deciduae. Similar results were obtained with decidual supernatants from days 11, 12 and 14 of pregnancy (data not shown). Additional data in this figure also indicate that the rabbit polyclonal anti-GM-CSF antibody was specific for GM-CSF, since it was unable to block response of DA-1 cells to IL-3 or erythropoietin, while completely blocking the proliferation of these cells in response to recombinant GM-CSF. The results indicate that the T cell-dependent growth factor present in decidual cell supernatants, which is produced in greater quantities in allogeneic than syngeneic deciduae, is GM-CSF, a lymphokine which we have previously identified as being a growth factor for trophoblast cells. These results provide clear evidence for spontaneous T cell activation at the maternal-fetal interface, with some of it due to the presence of fetal alloantigens and some due to as yet unknown mechanisms (C.F. allogeneic versus syngeneic GM-CSF levels).

DISCUSSION

In our initial attempts to evaluate the immunotrophism hypothesis (11,12) we selected allogeneically pregnant females as an experimental model to see whether deletion of maternal T cells in mid-gestation would affect placental proliferation and phagocytosis. We expected to find significant results if the hypothesis was correct, because the fetal placenta expresses foreign histocompatibility alloantigens which the maternal T cells can recognize (1). The results presented in this paper somewhat surprisingly extend this hypothesis into syngeneic pregnancy. Thus deletion of maternal T cells with either anti-CD4 or anti-CD8 monoclonal anti-T cell antibodies at mid-gestation of syngeneic pregnancy leads to a comparable reduction in placental proliferation, if one considers the uptake of tritiated thymidine per milligram of placental tissue. In addition, we obtained results which indicate that, while anti-CD8 antibody reduces the capacity of the placenta to phagocytose latex particles, anti-CD4 antibody does not have this effect, and this pattern holds true for both allogeneic and syngeneic pregnancies, suggesting that similar immune mechanisms operate in both circumstances. In order to begin to dissect this immunotrophic interaction between maternal T cells and fetal placental cells, we explored the capacity of decidual cell supernatants to stimulate the growth of placental cells *in vitro*. They did so, and surprisingly both allogeneic and syngeneic decidual cells had roughly comparable activity.

It was thus necessary to begin to identify the types of growth factors being released from these decidual cells at various stages of gestation. We therefore took advantage of the DA-1 cell line, whose lymphokine responsiveness has been characterized (15,17). The first observation obtained from applying decidual cell supernatants to this cell line was that those from allogeneic deciduae were more stimulatory than the supernatants from the syngeneic deciduae, and the ontogenic pattern corresponded roughly to the relative influx of T cells into the two types of the deciduae, as reported by others (2,3). This pattern includes an earlier influx of T cells in allogeneic as compared to syngeneic pregnancy, with the peak at 12 days for the former and 13 days for the latter, and a higher overall peak in the allogeneic case. This pattern is reflected in the decidual cell supernatant stimulatory effect on the DA-1 cells, as was shown in Figure 2. This suggests that the growth factors involved are released by T cells following their migration into the decidua. This possibility was strengthened by observing that maternal T cell deletion during pregnancy abolishes the capacity of both types of decidual cell supernatants to stimulate DA-1 cell lines.

The DA-1 cell line is responsive to a number of growth factors, including IL-3, GM-CSF and erythropoietin, but not CSF-1 (15,17). Since we had previously shown that GM-CSF and IL-3 are both capable of stimulating placental cell growth *in vitro* (12), we decided to determine whether either one of these lymphokines could account for for the growth factor activity found in decidual supernatants. GM-CSF was unambiguously identified as the primary source of this activity, because rabbit polyclonal anti-GM-CSF antibody almost completely abolished the capacity of decidual cell supernatants to stimulate the DA-1 cell line. This was true for both syngeneic and allogeneic deciduae, strengthening our conclusion from the T cell deletion experiments that maternal T cells, which make GM-CSF, are important in syngeneic as well as allogeneic placental proliferation. Thus these experiments support the idea that in both allogeneic and syngeneic pregnancy maternal T cell influx leads to a release of lymphokines, in particular GM-CSF, which can promote placental growth. It is interesting to note that more GM-CSF is found in allogeneic compared to syngeneic decidual cells, and this could potentially account for some of the difference between the size of allogeneic versus syngeneic placenta.

However, these experiments do not rule out the presence of other growth factors released from decidual cells during pregnancy. We have obtained preliminary evidence, using a radioreceptor assay, that CSF-1 is also present in the decidual cell supernatants from both allogeneic and syngeneic pregnancies. The DA-1 cell line is not sensitive to CSF-1 and so CSF-1 would not have been detected in the assays depicted in Figures 2, 3 or 4.

These experiments raise the question of which antigens are being recognized by maternal T cells. One likely candidate for the allogeneic situation is the Class I major histocompatibility complex antigens expressed by fetally-derived invasive trophoblast. However, the base line responsiveness seen in the syngeneic situation situation cannot be accounted for by such antigenic exposure. One possibility is that the maternal T cells are detecting the expression of unusual Class I antigens, which we and others have shown to be expressed at the mRNA level in the placenta (22). Alternatively, other mechanisms of an equally unusual sort can be invoked, such as a non-specific stimulation of T cells by trophoblast cells in a closed immunotrophic cycle. This important question must await future experimentation to be resolved in a satisfactory manner.

Another important point to be addressed is the physiological importance of the observations made in this paper. The data in Tables II and III indicate that deleting maternal T cells during mid-gestation had no discernible effect on fetal viability in the strain combinations employed here. This is not always the case. When comparable T cell depletion is done to CBA females pregnant by BALB/c, which ordinarily show a low rate of spontaneous abortion, this dramatically increases the proportion of resorbing fetuses at day 14 of gestation, and significantly reduces the placental and fetal weight of the surviving offspring at day 14 of gestation (Chaouat submitted).[4] In addition, one can negate the improvement in fetal survival seen in CBA females immunized with BALB/c white cells prior to the pregnancy by DBA/2 (23,24), if the maternal T cells are deleted during mid-gestation (Chaouat, in preparation). In this case, the fetal resorption rate remains high while placental and fetal weights are reduced. Thus there are strain combinations in which maternal T cell deletion does have a pronounced effect on fetal viability. This raises the issue of what effect the maternal T cells are having on the placenta to promote fetal viability in some instances but not in others. One possibility is to suggest that maternal T cells stimulate the placenta to provide an improved barrier between the maternal and fetal circulations for the avoidance of blood-born infection, which can in some circumstances harm the fetus. Such a suggestion is strengthened by recent work of Hammilton and Hamilton who showed that CBA females pregnant by DBA males will abort only if housed in a regular animal facility and not in a specific pathogen-free animal room, despite a common origin of the mice in both animal facilities (25).

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[4] Chaouat, G., E. Menu, I. Athanassakis and T.G. Wegmann, Maternal T cells regulate placental size and fetal survival (submitted for publication).

Finally, the experiments presented in this paper hint at the existance of a complex immunoregulatory network involving CD4 and CD8-positive T cells, somehow cooperating with each other to promote placental growth. The fact these two subsets of T cells have differential effects with respect to placental phagocytosis indicate that the interaction is a complex one. Not even addressed in the experiments presented here are the potential roles for other cells that have been described in the decidua, such as non-specific suppressor cells (26,27) and natural killer cells (28). Clearly, a long range goal for understanding this system must include cloning of the cells involved in this local immunoregulatory network with *in vitro* reconstruction of the various interactions which occur to regulate the growth of the placenta. Only in this manner will we begin to understand how the maternal immune system regulates placental growth and fetal survival. The experiments presented here provide a basis upon which to carry out that analysis.

Acknowlegments

We thank Bonnie Lowen for technical assistance.

Figure 1

Decidual cell supernatants stimulate placental cell proliferation *in vitro*. C3H/HeJ x BALB/cJ (allogeneic) or C3H/HeJ x C3H/HeJ (syngeneic) decidual cells derived from day 12 of pregnancy were tested for the ability of their culture supernatants to induce placental proliferation as described in materials and methods. Only supernatants collected on day one of culture are shown, since these gave the highest placental profileration in both cases. Recombinant GM-CSF (50 ng/ml) was used as a positive control in these experiments. The results are expressed as the mean value of triplicate cultures, and the error bars indicate one standard deviation. In control experiments, spleen cell supernatants prepared in a similar manner were unable to stimulate placental cell proliferation above background, as follows: spleen supernatants from C3H females mated with BALB males: 1,124 ± 160 cpm, spleen supernatants from C3H females mated to C3H males: 1,045 ± 192 cpm, medium control: 1051 ± 34 cpm.

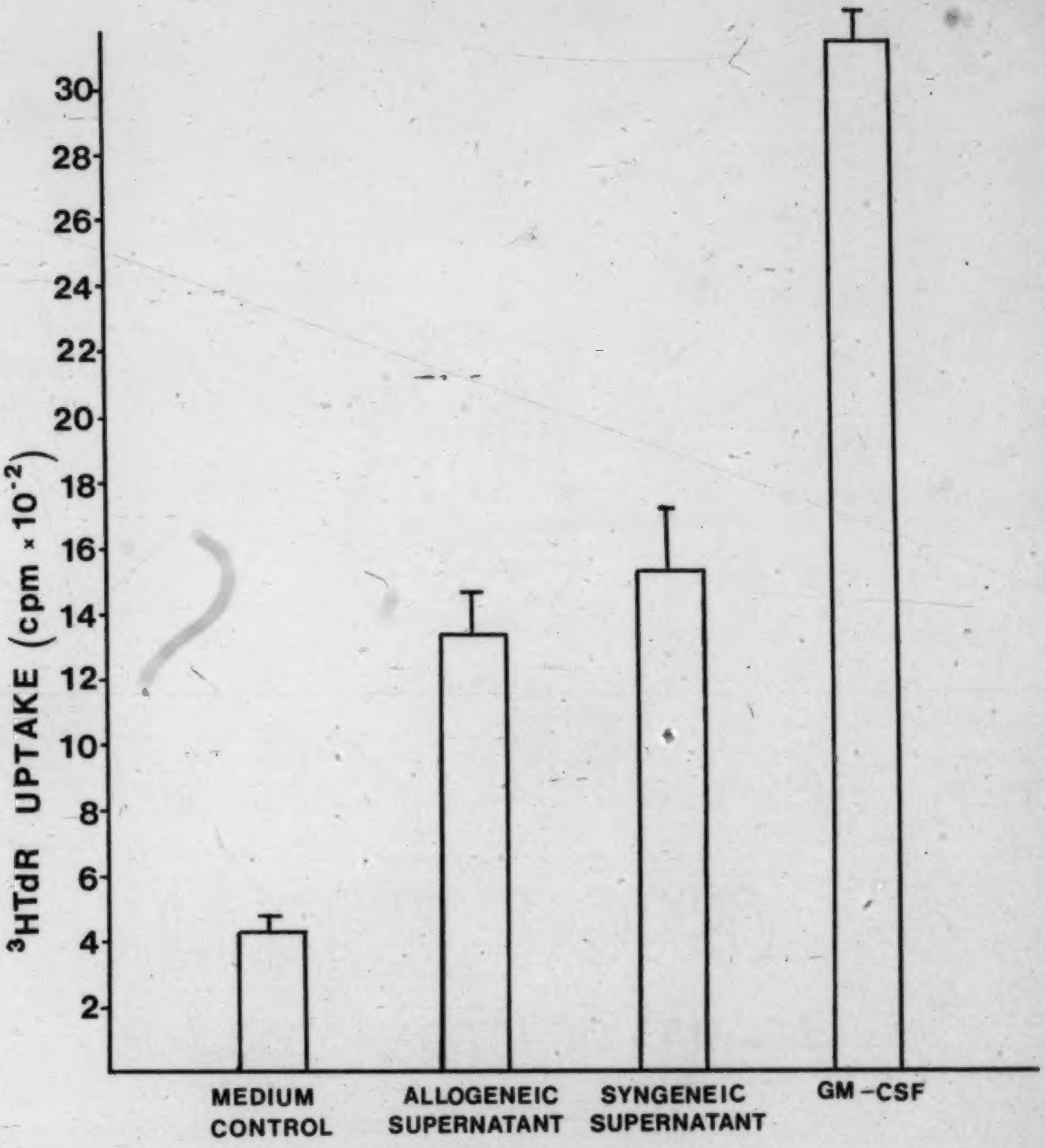
^{3}HTdR UPTAKE (cpm × 10^{-2})
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
MEDIUM CONTROL
ALLOGENEIC SUPERNATANT
SYNGENEIC SUPERNATANT
GM-CSF

Figure 2

Decidual cell supernatants contain growth factors that stimulate DA-1 cell proliferation. 10^4 DA-1 cells were cultured with decidual cell supernatants derived from BALB x C3H (allogeneic) or C3H x C3H (syngeneic) matings at 50% v/v, on days 11, 12, 13 and 14 of pregnancy. The amount of DA-1 proliferation was assessed 2 days later by adding 1 μCi of ^{3}HTdR per well. IL-3 and GM-CSF were used as positive controls in these experiments. IL-3 (300 U/ml) : 13,585 ± 1,745 cpm; GM-CSF (50 ng/ml) : 3,990 ± 346 cpm; medium control : 337 ± 108.

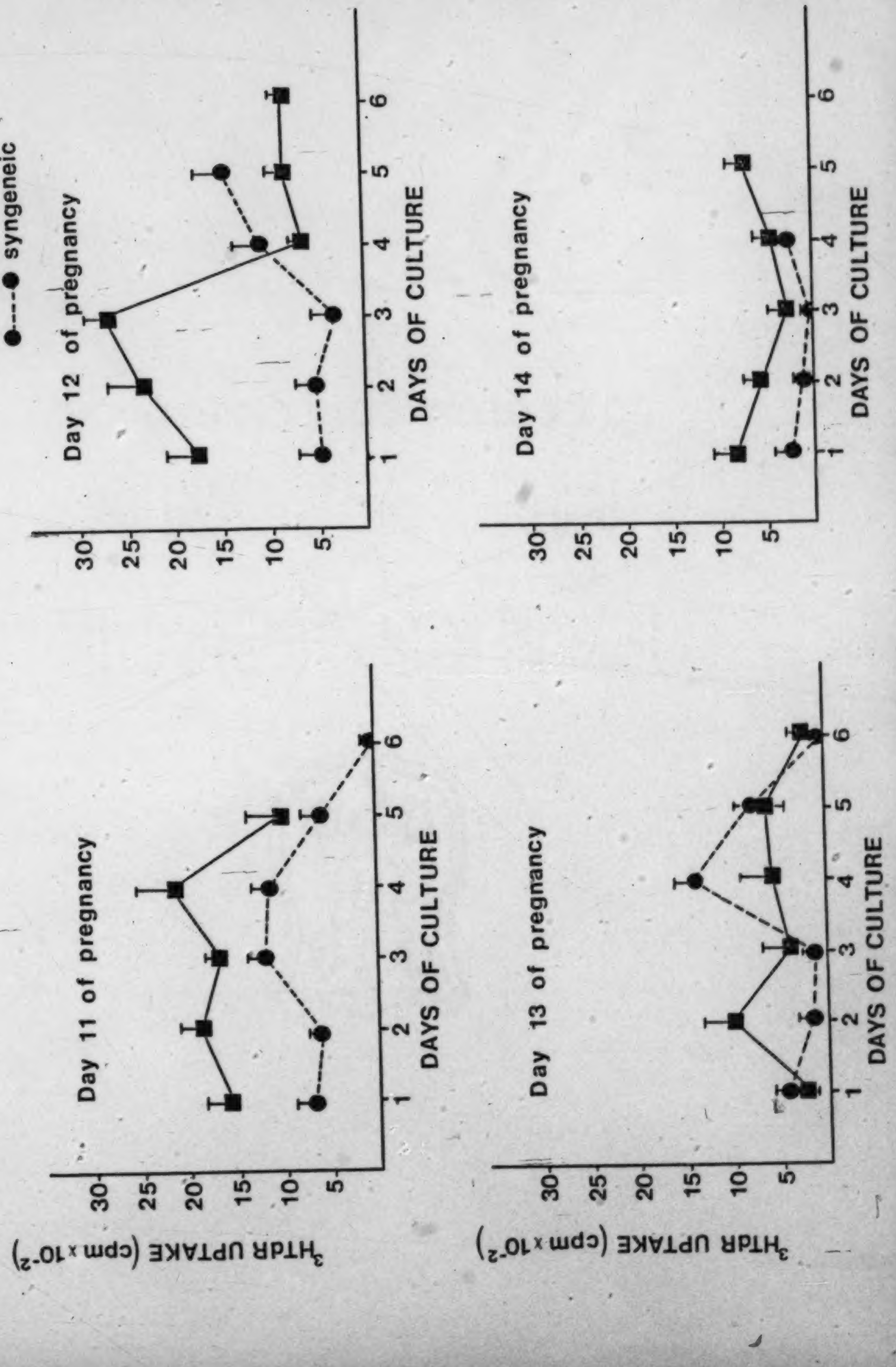
allogeneic
syngeneic
Day 11 of pregnancy
Day 12 of pregnancy
Day 13 of pregnancy
Day 14 of pregnancy
^{3}HTdR UPTAKE (cpm x 10^{-2})
30
25
20
15
10
5
DAYS OF CULTURE
1
2
3
4
5
6

Figure 3

Injection of allogeneically (x BALB) or syngeneically (x C3H) pregnant C3H females with anti-L3T4 and anti-Ly2.1 monoclonal antibodies on days 8, 10 and 11 of pregnancy reduces lymphokine production by decidual cells. Supernatants from day 12 decidual cell suspensions were tested for their ability to stimulate DA.1 cell proliferation. Only results from the peak response, corresponding to day 3 and 5 of culture for the allogeneic and syngeneic pregnancies respectively, are shown here. Identical results were obtained with all tested mice (3 mice per group). The results represent triplicate cultures ± SD. IL-3 (300 U/ml) was used as a positive control in these experiments. IL-3 :20,784 ± 2,146 cpm. Negative control : 590 ± 116.

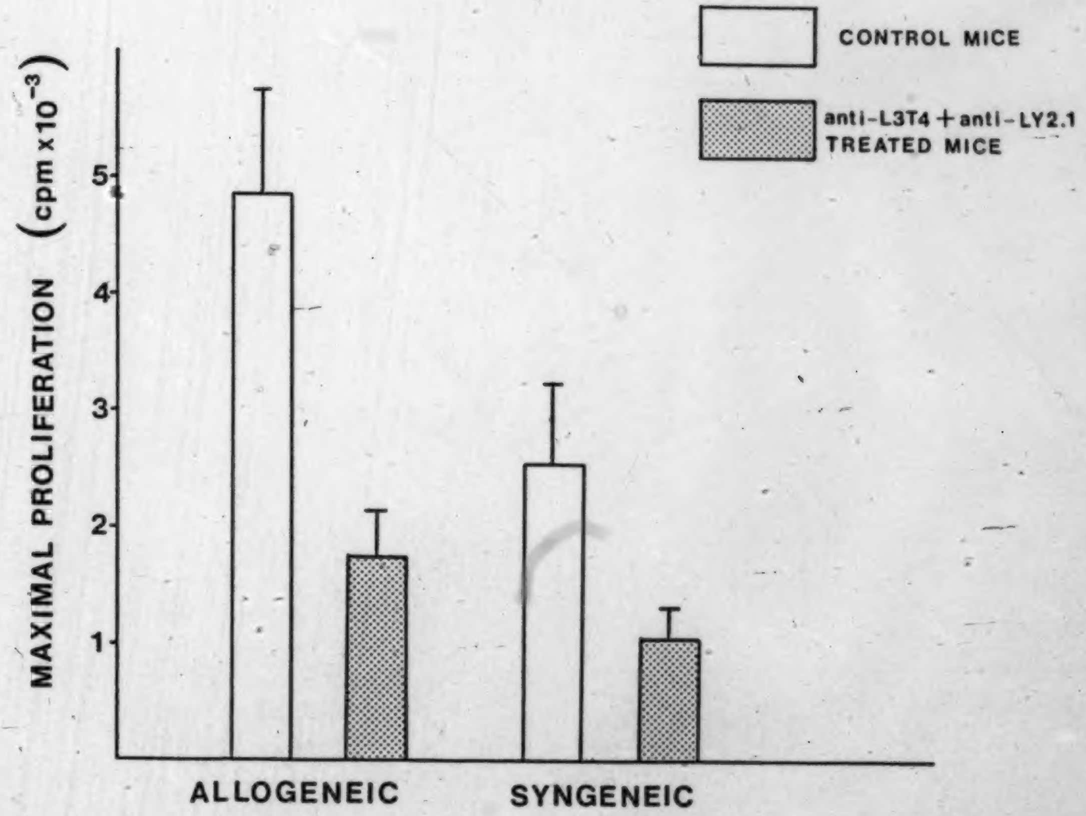
CONTROL MICE
anti-L3T4 + anti-LY2.1 TREATED MICE
MAXIMAL PROLIFERATION (cpm x 10^{-3})
5
4
3
2
1
ALLOGENEIC
SYNGENEIC

Figure 4

Rabbit anti-mouse GM-CSF antibody blocks the DA-1 stimulatory activity of allogeneic and syngeneic decidual supernatants. Decidual cell supernatants prepared from the deciduae of C3H x BALB (allogeneic) and C3H x C3H (syngeneic) matings at day 13 of pregnancy, were collected from days one to six of culture. They were pooled and tested for their ability to stimulate DA-1 cell growth, in the presence or absence of anti-GM-CSF antibody as described in the material and methods. Supernatants from decidual cells taken from days 11, 12 and 14 of pregnancy gave similar results (data not shown). GM-CSF (10 pg/ml) was used as a positive control, in order to the ascertain the specificity of the anti-GM-CSF antibody. This antibody was unable to block the response of DA-1 cells to IL-3 (300 U/ml) or human recombinant erythropoietin (EPO, 5 U/ml). The results represent triplicate cultures ± one standard deviation.

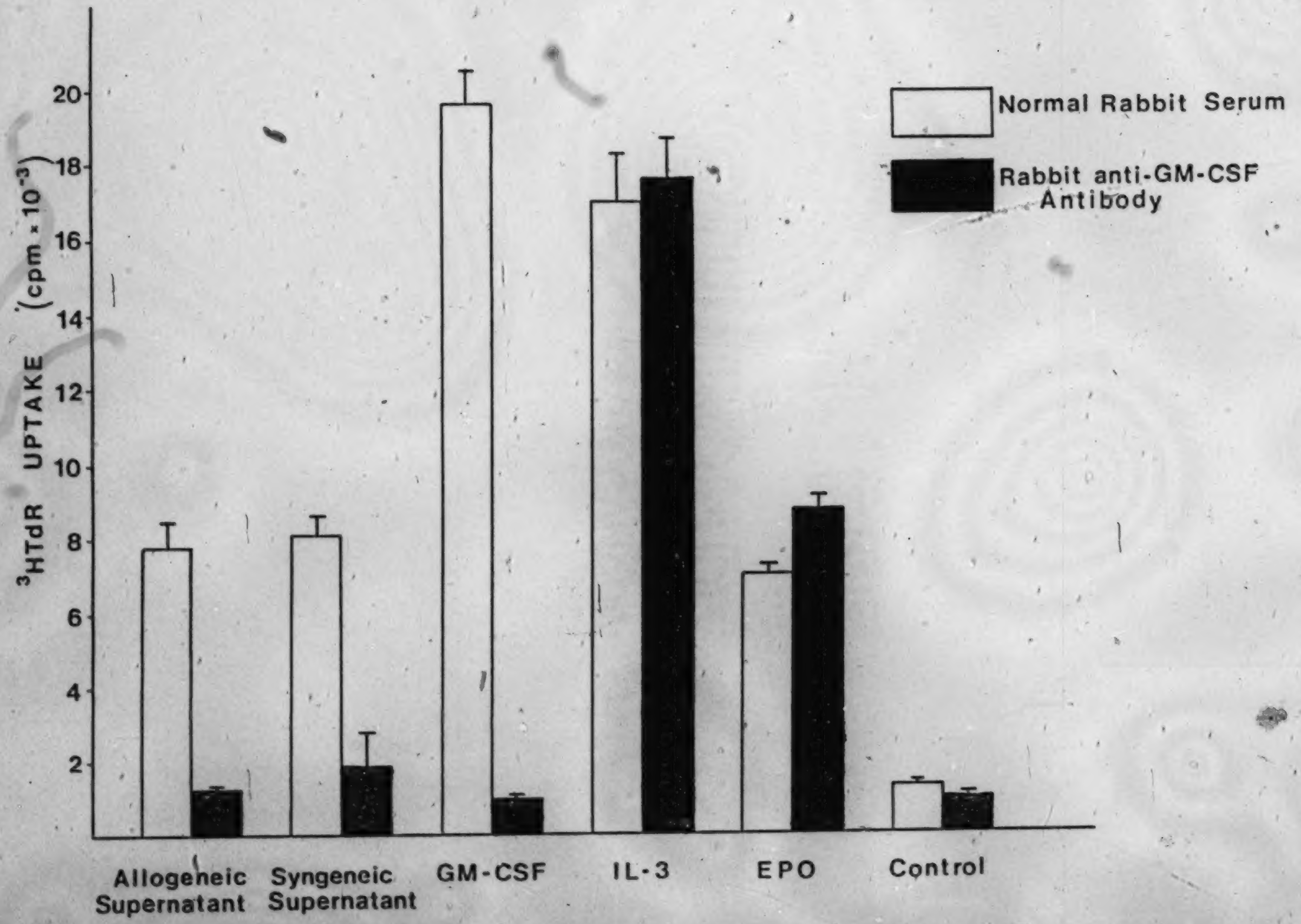
Normal Rabbit Serum
Rabbit anti-GM-CSF Antibody
^{3}HTdR UPTAKE (cpm × 10^{-3})
20
18
16
14
12
10
8
6
4
2
Allogeneic Supernatant
Syngeneic Supernatant
GM-CSF
IL-3
EPO
Control

TABLE I

Elimination of L3T4 and Ly2 positive cells in the spleen after *in vivo* treatment with anti-L3T4 and anti-Ly2.1 monoclonal antibodies.

Percent dead cells

	Depletion *in vivo* with:		
Treatment *in vitro* with:	--	anti-L3T4	anti-Ly2
anti-L3T4+C′	28±7	0	22±2
anti-Ly2+C′	22±4	13±5	1±1

Percent positive fluorescent cells

	Depletion *in vivo* with:		
Treatment *in vitro* with:	--	anti-L3T4	anti--Ly2
anti-L3T4+FITC*	22±2	0	31±2
anti-Ly2+FITC	14±8	18±3	0

* FITC labelled goat anti-rat IgG.

TABLE II

Effect of maternal L3T4 and/or Ly2.1 depletion on proliferation and phagocytosis in allogeneic[1] placentae

Treatment[2]	^{3}HTdR uptake (cpm/placenta)	^{3}HTdR uptake (cpm/mg)	Phagocytosis (% of placental cells)	placental weight (mg)	% of normal embryos
Anti-H-2k^{b}[3]	11,183±864	118±9	39±5	95±5	87±9
Anti-Ly2.1	6,357±1,393	70±15	12±5	91±8	83±10
Anti-L3T4	6,682±685	74±8	46±5	90±6	84±14
Anti-L3T4 + anti-Ly2.1	6,103±897	67±10	22±4	93±7	85±8

1. C3H/HeJ females were mated to BALB/cJ males and their placentae were assessed for proliferation and phagocytosis on day 12 of pregnancy.

2. Mice were injected i.p. with 0.5 mg of anti-L3T4 and/or 0.5 mg of anti-Ly2.1 on days 8, 10, and 11 of pregnancy. 5 to 7 pregnant mice, carrying at least 4 fetuses each, were tested in each case.

3. Anti-H-2k^{b} is a protein-A purified IgG2a monoclonal antibody produced by the 28.8 hybridoma. Untreated mice or mice injected with culture medium showed the same placental proliferation and phagocytosis as the inappropriate antibody-treated controls:

Proliferation:	11,057±574 and 10,563±1,022 cpm/placenta in the untreated and medium-injected controls respectively.
Phagocytosis:	38±2 and 34±5 percent of placental cells in the untreated and medium-injected controls, respectively.

TABLE III

Effect of maternal L3T4 and/or Ly2.1 depletion on proliferation and phagocytosis in syngeneic[1] placenta

Treatment[2]	^{3}HTdR uptake (cpm/placenta)	^{3}HTdR uptake (cpm/mg)	Phagocytosis (% of placental cells)	placental weight (mg)	% of normal embryos
Anti-H-$2k^b$ [3]	8,983±924	118±12	33±4	76±9	89±4
Anti-Ly2.1	4,797±625	74±10	21±2	65±4	72±2
Anti-L3T4	4,339±510	66±8	32±5	66±2	86±2
Anti-L3T4 + anti-Ly2.1	3,622±956	50±13	30±3	72±8	82±6

1. C3H/HeJ females were mated to C3H/HeJ males and their placentae were assessed for proliferation and phagocytosis on day 12 of pregnancy.

2. Mice were injected with 0.5 mg of anti-L3T4 and/or 0.5 mg of anti-Ly2.1 on days 8, 10, and 11 of pregnancy. 5 to 7 pregnant mice, carrying at least 4 fetuses each, were tested in each case.

3. The anti-H-$2k^b$ is a protein-A purified IgG2a monoclonal antibody produced by the 28.8 hybridoma. Untreated mice showed the same placental proliferation (8,751±1,421 cpm/placenta) and phagocytosis (74±11) as the inappropriate antibody-treated controls.

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CHAPTER V

CONCLUSIONS

Fetal survival in a semiallogeneic maternal environment is one of the most intriguing achievements of nature. It contrasts sharply with the rejection problems of artificial organ transplantation and raises questions concerning the mechanisms involved in successful allogeneic pregnancy. The approach to this issue points in three not mutually exclusive directions: (a) does the fetus protect itself? (b) does the maternal system protect the fetal allograft or (c) is cooperative interaction between the maternal and fetal systems important to a successful outcome? One possible answer to the first question has been formulated in the placental barrier hypothesis, according to which the placenta filters out any kind of immune effectors that could be harmful for the fetus (Chaouat et al. 1983). An answer to the second question is formulated in the immunosuppressive hypothesis, which postulates that the maternal immnune response is suppressed either systemically or locally, and more likely the latter, during pregnancy and thus rejection cannot take place (Nagarkatti and Clark 1983). The interactive nature of maternal and fetal systems has been highlighted by the immunotrophic hypothesis, which predicts that fetal alloantigens stimulate maternal T cells to secrete their lymphokine products, which promote placental growth and thus strengthen the placental barrier, which in turn improves fetal viability (Wegmann 1987). The present study has concentrated on evaluating predictions of the immunotrophic hypothesis. The results were found to conform to such predictions. In brief, the present study showed that the T cell-derived lymphokines IL-3 and GM-CSF as well as the cytokine CSF-1 stimulate placental proliferation and phagocytosis *in vitro*. The proliferating placental polulation was shown to be fetal in origin, adherent, phagocytic and non-specific esterase positive. Subsequent marker analysis characterized these cells as trophoblast-macrophages and distinguished them from placental macrophages. Furthermore, it was shown that maternal T cell depletion decreases placental proliferation and phagocytosis *in vivo*. Finally, the decidua was identified as a tissue able to generate lymphokines that can stimulate placental cell proliferation *in vitro*.

T Cell Lymphokines Stimulate Trophoblast Growth

The direct approach to examine the immunotrophic hypothesis was to inquire whether T cell-derived lymphokines can stimulate trophoblast growth *in vitro*. Indeed it was found that column purified as well as recombinant IL-3 and GM-CSF could increase ^{3}HTdR uptake of placental cells as shown by direct counting of beta emission as well as by autoradiography. Similar placental stimulation was seen with the cytokine CSF-1. Isozyme analysis revealed that the proliferating cells were fetal in origin. When these cells were examined at least 48 hours after culture, they were shown to be adherent, phagocytic and non-specific esterase positive. We have operationally designated them Fetal Adherent Phagocytic Placental cells (FAPP cells). These observations suggested that FAPP cells are either trophoblasts or placental macrophages. Further characterizaton of these cells using surface marker analysis showed that FAPP cells are trophoblasts by the criteria of being cytokeratin-positive and vimentin-negative, and macrophages by the criteria of being F4/80 and Mac-1 positive. Double staining experiments showed that the same cell bears both types of markers. More evidence suggesting the macrophage character of these FAPP cells was obtained with CSF-1 binding experiments. CSF-1 is a growth factor whose actions and receptor were hitherto considered limited to mononuclear phagocytes. We found that CSF-1 bound to FAPP cells with the same manner as it does to macrophages. Other investigators have detected the transcript of the CSF-1 receptor gene on the trophoblastic cell line BeWo (Muller et al. 1983). This is the first time that epithelial-type cells were shown to express markers that until now were thought to be specific for macropahges. These observations allow us to postulate that at least some of the trophoblast cells are large macrophage-like cells which can filter out agents (either maternal or microbial), that could be harmful for the fetus, thus fortifying the placental barrier. In addition, FAPP cells do not express class II MHC antigens, which are necessary for antigen recognition and generation of CTLs or other types of immune response harmful to the fetus. In order to characterize them further, dissociated placental cells were separated in a percoll density gradient step gradient. The vast majority of FAPP cells were were isolated from the 1.02-1.04 g/ml interface (FAPP40). They also represented the majority of isolated placental cells, showed surface markers characteristic of both macrophage and trophoblast and responded to GM-CSF. This separation revealed a minor cell population with only macrophage characteristics at the 1.04-1.06 g/ml interface (FAPP60). The number of these FAPP60 cells (5-10% of the total population) corresponded to the numbers of placental macrophages as described by other investigators (5-7%, Wood 1980, Bulmer and Johnson 1984). Another cell population was isolated from the 1.06-1.08 g/ml interface. Although this was maternal in origin and non-phagocytic, it proliferated in response to GM-CSF.

The fact that trophoblast-macrophages appear to in the 1.02-1.04 g/ml percoll interface allowed us to determine whether they originate from the labyrinthine trophoblast or spongiotrophoblast zone. Separation by dissection of the two layers prior to the density step separation showed that FAPP40 cells are present in both trophoblast layers. The same is true for FAPP60 cells. However, the cells of maternal origin at the 1.06-1.08 g/ml interface were present only in the spongiotrophoblast zone. FAPP60 cells in both layers show autonomous growth which increased 2-fold in the presence of GM-CSF and CSF-1. FAPP cells isolated from dissected trophoblast grew very poorly in culture if not stimulated. Although CSF-1 induced a 14-fold increase in FAPP40 cell proliferation, the net ^{3}HTdR uptake did not approach that seen with FAPP60 cells. This behavior of FAPP40 cells argues for the presence of a growth regulatory network between the different subpopulations in the placenta. This is because unseparated FAPP cells grow much better in culture than percoll-separated FAPP cells in the presence of lymphokines (compare results in pages 86, 136 and 143).

In general these results showed that at least some trophoblasts proliferate in response to the T cell-derived lymphokines IL-3 and GM-CSF. In addition they bind and respond to the macrophage-specific growth factor CSF-1.

The trophoblast cells that are lymphokine stimulated express macrophage specific markers, which allowed us to refer to them as trophoblast-macrophages. The realization of the full growth potential of these cells requires the presence of placental macrophages and/or other placental cell populations. Placental cell supernatants have been shown to contain CSFs that stimulate monocytes, which in the intact placenta could stimulate either macropahges or trophoblasts (Toder et al. 1987). This is a further indication of the presence of a regulatory network between different cell populations in this tissue. Macrophages could be producing PDGF (Martinet et al. 1986) CSF-1, and/or IL-1, which could act on trophoblast cells. Trophoblasts could also secrete growth factors stimulating macrophages and other placental populations. Indeed, lymphokine-stimulated long term cell lines from placental cells, as well as their culture supernatants, were shown to non-specifically stimulate a variety of T cell lines to proliferate in the absence of antigen, antigen-presenting cells or both (Mogil and Wegmann 1988). In addition, it has been reported that exposure of human trophoblast to peripheral blood lymphocytes increases human chorionic gonadotropin synthesis (Dickman and Cauchi 1978, Kaplan 1983). Furthermore, sex steroid hormones can stimulate CSF-1 production by the uterine glandular epithelial cells, which in turn can activate trophoblast cells as well as macrophages (Pollard et al. 1987). All these observations argue for the presence of cooperative interactions between the maternal and fetal systems important to a successful outcome.

Maternal T Cell Deletion Decreases Placental Proliferation and Phagocytosis in vivo

A second prediction is that T cell deletion from the gestating female decreases placental growth *in vivo*. Therefore, the effect of maternal T cell depletion on placental proliferation and phagocytosis *in vivo* was examined. Polyclonal antibody depletion of maternal T cells using rabbit anti-mouse thymocyte serum at mid-gestation reduced placental proliferation and phagocytosis *in vivo*. Although it was shown that this effect correlated with the elimination of maternal T cells, monoclonal antibody depletion was performed to exclude the possibility of any non-specific effects of the antibody, and to examine possible subset interactions. Treatment of the mother with anti-CD4 (L3T4) and anti-CD8 (Ly2.1) monoclonal antibodies treatment resulted in a 50% reduction in proliferation and phagocytosis of the placental tissue *in vivo*. Single monoclonal treatment showed that both anti-Ly2.1 and anti-L3T4 decreased placental proliferation, but only anti-Ly2.1 decreased phagocytosis in the placenta. These results show either that CD4 and CD8 T cells act on different cell populations, or they have a different effect on the same placental cells (different growth factors secreted by each subset). However, the fact that no additive effect was observed when anti-CD4 and anti-CD8 were simultaneously injected into the gestating females, is in favor of the latter interpretation. The monoclonal antibody treatment showed the same effect in syngeneic and allogeneic pregnancies. Although the results were more prominent in the allogeneic situation, an identical amount of inhibition of proliferation per unit of tissue was obtained in both cases.

Treatment with the above monoclonal antibodies in the strain combinations tested here, did not decrease placental weight or fetal viability. Nevertheless, when it was applied to the CBAxDBA/2 abortion model, it had a significant effect. This treatment was able to reverse the effect of the anti-abortion immunization of CBAxDBA/2 females with BALB/c cells; i.e., it increased abortion and decreased placental and fetal weight as if no prior immunization was performed. In addition maternal T cell deletion of CBA females pregnent by BALB/c males dramatically increased the percent of resorbed embryos and significantly reduced their placental and fetal weight. Furthermore, this treatment causes a considerable decrease in placental and fetal weight in MRL autoimmune mice, which are known to produce large placentae (Chaouat et al. 1988). These experiments were performed as a direct result of the work presented in this thesis.The results presented in this study show that although the presence of an immunotrophic response is not obvious in all strain combinations (as to differences in placental weight and fetal survival), it can be proved in terms of placental cell proliferation and function.

Placental.Growth is Locally Stimulated by Decidua-derived Growth Factors

A final prediction of the initial hypothesis is that the relevant lymphokines should be present in the decidua, and more so in allogeneic versus syngeneic pregnancy. In order to test this prediction, supernatants from decidual cells or spleen cells were collected and tested for their ability to induce placental cell proliferation and/or DA-1 cell proliferation. DA-1 cells respond most strongly to IL-3 and GM-CSF. Since these are the two lymphokines which we had previously identified as capable of stimulating placental proliferation, we concentrated our study on these T cell products. Only decidual cell supernatants, and not spleen cell supernatants, were able to stimulate placental and DA-1 cell proliferation. On a per cell basis, decidual cell supernatants from days 11 and 12 of pregnancy showed the highest growth factor content. This agrees with the results of others in which lymphocytes first appear in the decidua on day 10, peak on day 12 and decline thereafter until day 15, when a second migration occurs (Lala et al. 1986). Allogeneic decidual supernatants were able to induce higher levels of proliferation of DA-1 cells than syngeneic decidual supernatants. The growth factor contained in these supernatants, capable of stimulating DA-1 cell growth, was dependent upon maternal T cells since monoclonal T cell depletion of pregnant females resulted in a 60% decrease of DA-1 cell proliferation in both the allogeneic and syngeneic cases. The lymphokine contained in the decidual supernatants was shown to be GM-CSF, since antibody to GM-CSF completely blocked the stimulatory ability of the decidual preparations on DA-1 cells. However, these assays can only detect the presence of GM-CSF and IL-3. The fact that syngeneic and allogeneic preparations give the same degree of stimulation on placental cells, argues for the existance of factors other than T cell-derived lymphokines in decidual supernatants which stimulate placental growth. From previous results we know that CSF-1 is able to stimulate placental cell proliferation. In addition it was shown that a semi-purified suppressor factor produced by non-T (null) cells in the decidua, first described by Clark et al. (1980), could increase placental cell proliferation and phagocytosis *in vitro*. Since neither

CSF-1 nor the decidua-derived suppressor factor are capable of inducing DA-1 cell proliferation (data not shown), one cannot exclude the possibility that these factors are also present in the decidual cell preparations. Indeed, CSF-1 activity was detected in both allogeneic and syngeneic decidual supernatants since both were able to compete with ^{125}I-CSF-1 in a radioreceptor assay. This agrees with observations of Stanley and his collegues who described that the uterine levels of CSF-1 increases during pregnancy in a hormone-dependent manner (Bartocci et al. 1986, Pollard et al. 1987).

In conclusion, the results presented in this thesis support the placental immunotrophism hypothesis in three major ways. Maternal T cells are stimulated by placental and/or fetal antigens (Billington 1986) to secrete GM-CSF which stimulates both trophoblast-macrophage proliferation and phagocytosis, which in turn strengthens the placental barrier and improves fetal viabilty. At the placental cellular level, the results presented here indicate the existance of a regulatory network, in which maternal T cells, null cells, placental macrophages and trophoblasts interact through their products to improve fetal survival. Two future projects are apparent from this work. First, it will be necessary to clone the T cells in the decidua, define their antigen specificity and characterize the lymphokines produced after stimulation with the placental fetal antigens. Second, one must also analyze the regulatory network in the placenta, with respect to growth factor production and cellular interaction. The interactions between decidual cells, and the different placental populations, including trophoblasts, macrophages and other yet non-characterized cells, are no doubt complex and will require a major effort to unravel. Yet, this will be necessary in order to understand in detail how maternal immune cells participate in placental growth, and therefore fetal survival. Most importantly, the work presented in this thesis indicates the maternal immunotrophic response to be impotrant to placental growth and fetal survival and provides clear direction in how to proceed with an analysis of this response.

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